This Number Completes Volume 20
Volume 20 December 1956 Supplement

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

CONTENTS

Published by the Agricultural Chemical Society of Japan
c/o Faculty of Agriculture, University of Tokyo

THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN
(NIPPON NOGEI KAGAKU KAI)

The Bulletin of the Agricultural Chemical Society of Japan is published quarterly to provide original papers on agricultural chemistry.

Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $ 6.00 for all oversea subscribers including postage fee. Please address orders to the sole agent.

The Agricultural Chemical Society of Japan also published the Journal of the Agricultural Chemical Society of Japan (Nippon Nogei Kagakukaishi), printed in Japanese.

The Agricultural Chemical Society of Japan

Sole Agent
Maruzen Co., Ltd.
P. O. Box 605, Tokyo Central
Tokyo, Japan.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 223~228]

Studies on the Oxidation of Terpenes with Ozone

Part VI. Limonene Monozonide and the Oxidative Decomposition

By Shin-ichi KATAOKA* and Yoshio HANADA

Camphor Research Department, The Central Research Institute, Japan Monopoly Corporation, Tokyo

Received March 14, 1956

The authors have been studying the addition of ozone to terpenes and the oxidation products of terpenes with ozone. In this report, β-isopropenyl-δ-acetyl-*n*-valeric acid and aldehyde were obtained by the oxidative decomposition of limonene monozonide with chromic oxide. A small quantity of hexahydro-*p*-acetyltoluene was also obtained by hydrogenation of the neutral part. It was first presumed that the main component of limonene monozonide was limonene-1,2-monozonide, but a small quantity of limonene-8,9-monozonide was also produced when limonene was ozonized in glacial acetic acid.

Nerescheimer[1] far back in 1916, reported that diacetylvaleric acid (III) is obtained by the oxidative decomposition of limonene diozonide with chromic oxide. Harries and Adam[2], however, presumed that (III) was unstable and gave cyclic compounds (IV or IV′). Furthermore, Harries and Nerescheimer[3] reported that ketozonide was given by the heating of limonene diozonide in acetic acid for a short time, and also that diketoaldehyde (II) was given by heating for twenty-four hours.

In 1940, Wakatsuki[4] obtained *p*-cymene, ketone and much resinous substance by the heating of ozonide in an aqueous solution followed by steam distillation.

Since 1950, the authors have reported several studies on the oxidation of limonene[5], pinene[6], terpineol[7], menthene[8] and limonene monohydrochloride[9] with ozone. In the previous paper[5], limonene was ozonated for half an hour to sufficient saturation. After the heating of ozonide in an aqueous solution followed by steam distillation, *p*-cymene, tetrahydro-*p*-acetyltoluene and ketone (semicarbazone m.p. 200–201°) were obtained in the volatile by-products. Two kinds of ketone, a crystal (m.p. 120°) and an acid were obtained in the nonvolatile products, but these main components could not be determined sufficiently.

In this report, acid and aldehyde obtained by the oxidative decomposition of limonene monozonide with chromic oxide were determined to be β-isopropenyl-δ-acetyl-*n*-valeric acid (VI) and β-isopropenyl-δ-acetyl-*n*-valeric aldehyde (IX), because (VI) was obtained by the oxidation of (IX) with silver oxide and β-isopropyl-δ-acetyl-*n*-valeric acid (VII) was obtained by the hydrogenation of (VI). It was first presumed that ozone was mainly absorbed to the 1,2-double bond of limonene for the first time and diozonide was gradually given.

It is also presumed that a small quantity of limonene-8,9-monozonide (X) is given as a by-product of limonene-1,2-monozonide (V), because hexahydro-*p*-acetyltoluene was

* Present address: Kagoshima Regional Bureau, Japan Monopoly Corporation, Kagoshima.

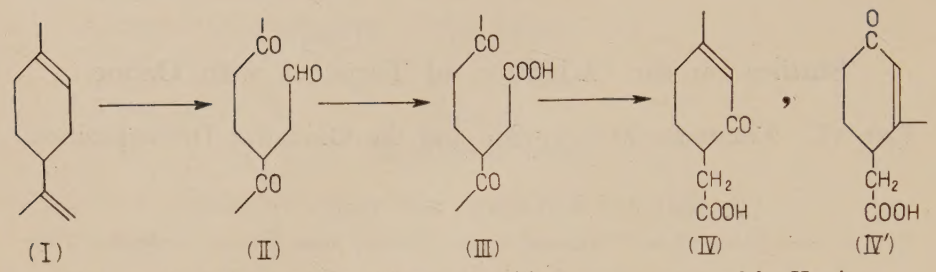
1) H. Nerescheimer, *Chem. Zentbl.*, **87**, II, 993 (1916).

2) C. Harries and H. Adam, *Ber.*, **49**, 1034 (1916).

3) C. Harries and H. Nerescheimer, *C.A.* II, 3238 (1917).

4) K. Wakatsuki, *Rept. of Camphor Engineers Association*, **8**, 29 (1940).

5)–9) S. Kataoka and Y. Hanada, *Shōnō-Kenkyū* (*Rept. of Camphor Research Dept., The Central Research Inst., J.M.C.*) **2**, 7 (1950), **3**, 29, 36 (1951), **4**, 22, 28 (1952).



(I) (II) (III) (IV) (IV′)

FIG. 1. Oxidation Products of Limonene with Ozone as presumed by Harries.

obtained in the lower boiling part of the hydrogenated neutral part.

RESULTS AND DISCUSSION

Wave numbers of 900 and 1650 cm^{-1} in the infrared spectrum in Fig. 2 (1) of the acid which was obtained by the oxidative decomposition of limonene monozonide with chromic oxide, indicated the presence of the $R_1R_2C=CH_2$ form double bond. The acid gave semicarbazone in a good yield and analytical data agreed with the theoretical value of β-isopropenyl-δ-acetyl-*n*-valeric acid (VI). As indicated in Table I, hydrogenation of the acid with palladium black gave a similar oil with β-isopropyl-δ-acetyl-*n*-valeric acid (VII), formally obtained by the oxidation of *p*-menthene-1[8] with ozone. Both infrared spectra agreed well with each other, as indicated in Figs. 2 (2), (3). Semicarbazones of the acid and the ethyl ester did not depress melting points by admixture with each other. According to these points, it is ascertained that this is (VII), and the acid obtained by the decomposition of limonene monozonide is (VI).

Infrared spectrum of ketoaldehyde fraction obtained by the distillation of the neutral part is shown in Fig. 2 (4). Wave numbers of 892 and 1650 cm^{-1} indicated the presence of the same double bond with (VI), and the wave number of 1717 cm^{-1} indicated the presence of the carbonyl group. Analytical value of the semicarbazone given in a good yield almost agreed with the theoretical value of β-isopropenyl-δ-acetyl-*n*-valeric aldehyde (IX).

Oxidation of this aldehyde with silver oxide and sodium hydroxide gave the same acid with (VI), and the melting point was not depressed by admixture with each other. As indicated in Table II, hydrogenation of this aldehyde gave a similar oil with that obtained by the oxidation of *p*-menthene-1[8]. The hydrogenated aldehyde gave semicarbazone (m.p. 208–209°). Infrared spectrum is shown in Fig. 2 (5), and the melting point of the semicarbazone was not depressed by admixture with each other.

Perfumery oil (semicarbazone m.p. 157–159°) was obtained in the lower boiling part of the hydrogenated neutral part. The melt-

TABLE I

PHYSICAL PROPERTIES OF β-ISOPROPYL-δ-ACETYL-*n*-VALERIC ACID AND THE ETHYL ESTER

Samples	b.p. (°C/mm)	d_{25}^{25}	n_D^{25}	Semicarbazone (°C)	Oxim (°C)	Ethyl ester b.p. (°C/mm)	Ethyl ester d_{25}^{25}	Ethyl ester n_D^{25}
The literature[10–12]	174–180/9	d^{20} 1.019	n_D 1.4566	152–153 158	75–80	143–146/12	—	—
Authors' from *p*-menthene-1[8]	145–153/2	1.0257	1.4580	158–159	75–77	137–139/12	0.9730	1.4442
This report	155/1	1.0229	1.4628	159–160	—	137–141/12	0.9702	1.4467

10) S. Kataoka, 8th Annual Meeting of Chem. Soc. Japan, Univ. of Tokyo, Apr. 3, (1955).

11) A. Baeyer and E. Oehler, *Ber.*, **29**, 31 (1896).

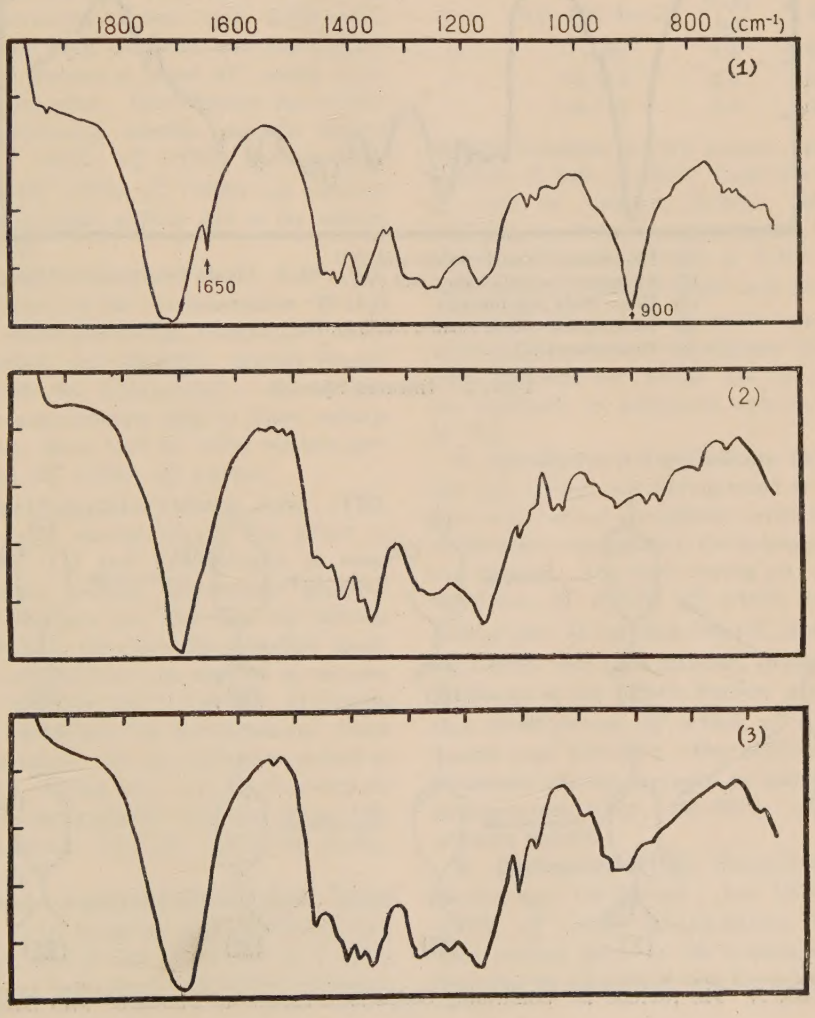
12) F.W. Semmler, *ibid.*, **40**, 2961 (1907).

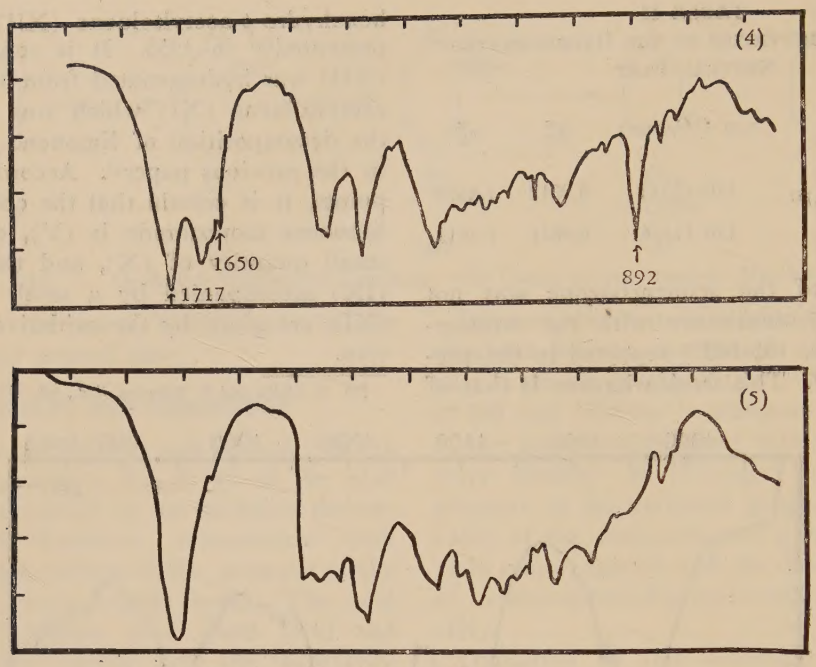
TABLE II
PHYSICAL PROPERTIES OF THE HYDROGENATED NEUTRAL PART

Samples	b.p. (°C/mm)	d_{25}^{25}	n_D^{25}
Authors' from *p*-menthene-1[8]	110–115/5	0.9812	1.4619
This report	110–115/4	0.9821	1.4613

ing point of the semicarbazone was not depressed by admixture with the semicarbazone (m.p. 161–162°) reported in the previous paper[8]. This semicarbazone is that of hexahydro-*p*-acetyltoluene (XII) as already presented[13] in 1955. It is considered that (XII) was hydrogenated from tetrahydro-*p*-acetyltoluene (XI) which was obtained by the decomposition of limonene monozonide in the previous paper[5]. According to these points, it is certain that the constitution of limonene monozonide is (V), containing a small quantity of (X), and that (VI) and (IX) accompanied by a small quantity of (XI), are given by the oxidative decomposition.

13) F. Makla and F. Tiemann, *ibid.*, **33**, 1938 (1900).





(1) β-Isopropenyl-δ-acetyl-*n*-valeric acid (VI)
(2) β-Isopropyl-δ-acetyl-*n*-valeric acid (VII)
(3) Ditto. (from *p*-menthene-1)
(4) β-Isopropenyl-δ-acetyl-*n*-valeric aldehyde (IX)
(5) Hydrogenated (IX)

FIG. 2. Infrared Spectra.

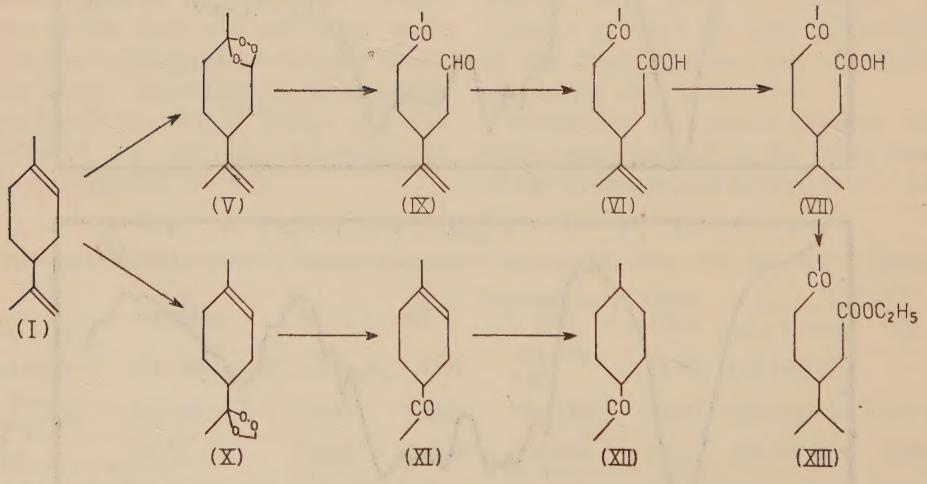


FIG. 3. The Method for Confirming Oxidation Products of Limonene with Ozone.

EXPERIMENTAL

1. Limonene. Tōhi oil (orange peel oil) was steam distilled, and vacuum distilled twice on a metallic sodium. Packed column: reflux ratio 1 : 20, number of theoretical plate ca. 18, was used. Properties of the distilled limonene are as follows: b.p. 79.5°/30 mm, d_4^{25} 0.8397, n_D^{25} 1.4699, $[\alpha]_D^{17}$ +119.03°.

2. Ozonization. Limonene (30 g) in glacial acetic acid (450 g) was ozonated by an ozone stream (oxygene pass 120 l/hr, concentration of ozone 29.55 g/m³, yield of ozone 3.546 g/hr) for three hours to the addition of 1 mol ozone. At this time, it gave white deep smoke. This solution was poured into a three-necked flask, cooled under 20°, and added acetic acid (1 l) solution containing chromic oxide (14 g) under stirring. After two days, the acetic acid was distilled under a reduced pressure at about 50°, added water and extracted with ether. Ether solution was washed by 3% sodium hydroxide solution, and the neutral part 11.8 g (d_4^{25} 1.0227, n_D^{25} 1.4790) was obtained. Acidic part 5.2 g (d_4^{25} 1.0566, n_D^{25} 1.4685) was obtained by the addition of dilute sulfuric acid to the sodium hydroxide solution.

3. β-Isopropenyl-δ-Acetyl-*n*-Valeric Acid (VI). The melting point of the semicarbazone (0.42 g) obtained from acidic part (0.5 g), recrystallized thrice from ethyl alcohol, was 182–183°. *Analysis* Found: N, 17.51. Calcd. for $C_{11}H_{19}O_3N_3F_1$: N, 17.43%. Heating of the semicarbazone (2 g) in dilute sulfuric acid (20 ml) on a steam bath for thirty minutes gave the original acid (d_4^{25} 1.0483, n_D^{25} 1.4781).

4. β-Isopropyl-δ-Acetyl-*n*-Valeric Acid (VII). (VI) (3 g) in ethyl alcohol (50 ml) was added to palladium black (1 g) and hydrogenated at room temperature under normal atmospheric pressure. After 1 mol of hydrogen was absorbed, the solution was filtered off from the recovered palladium black, the filtrate was concentrated and distilled in vacuum. The obtained saturated acid (1.7 g) (b.p. 115/1 mm, d_4^{25} 1.0199, n_D^{25} 1.4628) gave the semicarbazone. After recrystallization thrice, the semicarbazone melted at 159–160° and the melting point was not depressed by admixture with the authentic specimen (m.p. 158–159°). *Analysis* Found: N, 17.42. Calcd. for $C_{11}H_{21}O_3N_3$: N, 17.28%.

5. β-Isopropyl-δ-Acetyl-*n*-Valeric Acid Ethyl Ester (VIII). (VII) (5.0 g) in ethyl alcohol involving 3% hydrochloric acid (15 ml) was heated on a steam bath for two hours, neutralized with sodium carbonate solution and distilled. The semicarbazone of the obtained ethyl ester (4.2 g) (d_4^{25} 0.9674, n_D^{25} 1.4467) melted at 90–92°, and the melting point was not depressed by admixture with the authentic specimen (m.p. 92–93°).

6. β-Isopropenyl-δ-Acetyl-*n*-Valeric Aldehyde (IX). Neutral part (11.8 g) was distilled as shown in Table III (residue 2.2 g), and fraction No. 2 was redistilled. The redistilled fraction (b.p. 90–100°/2mm, d_4^{25} 1.0067, n_D^{25} 1.4783) gave the infrared spectrum as shown in Fig. 2 (4) and bromine was well absorbed. This fraction (0.5 g) gave semicarbazone (0.38 g) (m.p. 193–194°) insoluble in ethyl alcohol. *Analysis* Found: C, 51.07; H, 6.69; N, 29.70. Calcd. for $C_{12}H_{22}O_2N_6F_1$: C, 51.06; H, 7.80; N, 29.79%. Addition of the neutral part to Tollen's reagent ($AgNO_3$ 10 g, NaOH 5g, 50% methyl alcohol 150 ml) at 5–10° for one hour under stirring, gave the acidic part (2.6 g) d_4^{25} 1.0644, n_D^{25} 1.4747) followed by the neutrel part (2.4 g). The melting point of the semicarbazone (0.35 g) obtained from the acidic part (0.5 g) was 182–183°, and was not depressed by admixture with the semicarbazone of (VI).

TABLE III

FRACTIONAL DISTILLATION OF THE NEUTRAL PART

No.	b.p. (°C/3mm)	Yield (g)	d_4^{25}	n_D^{25}
1	– 95	1.8	0.9994	1.4759
2	95–115	5.6	1.0176	1.4760
3	115–140	2.2	1.0396	1.4788

7. Hexahydro-*p*-Acetyltoluene (XII). The neutral part (11.6 g) was hydrogenated at room temperature under normal atmospheric pressure. After 1 mol of hydrogen was absorbed, the hydrogenated substance was distilled. The lower boiling part (4.1 g) (b.p. 70–95°/3 mm, d_4^{25} 0.9612, n_D^{25} 1.4543) and the higher boiling part (5.7 g) (b.p. 95–115°/3 mm, d_4^{25} 0.9873, n_D^{25} 1.4639) were then obtained, (residue 1.0 g). Redistillation of the former fraction gave the fraction (b.p. 60–80°/5 mm, d_4^{25} 0.9226, n_D^{25} 1.4464) (semicarbazone m.p. 157–159°). This melting point of semicarbazone was not depressed by admixture with the semicarbazone (m.p. 161–162°), obtained in the previous paper[5].

8. Hydrogenated (IX). Redistillation of the latter fraction gave the fraction: b.p. 100–115°/4 mm. d_4^{25} 0.9792, n_D^{25} 1.4613; (semicarbazone m.p. 208–209°). This melting point of the semicarbazone was not depressed by admixture with the semicarbazone (m.p.

208–209°), obtained by the oxidation of *p*-menthene-1 with ozone in the previous paper[8].

The authors are grateful to Dr. M. Kitajima, Chief of the Camphor Research Department for his kind encouragement and Mr. T. Takeshita for his assistance with infrared spectra. They are also indebted to the Laboratory of Microanalysis, Department of Agric. Chemistry, Univ. of Tokyo for their microanalyses.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 229~235, 1956]

Studies on the Oxidation of Terpenes with Ozone

Part VII. Oxidative Decomposition of Limonene Diozonide*

By Shin-ichi KATAOKA** and Yoshio HANADA

Camphor Research Department, The Central Research Institute, Japan Monopoly Corporation, Tokyo

Received March 14, 1956

In order to prove the structure of cyclic keto-acid obtained from limonene diozonide on oxidation with chromic oxide, esterification, hydrogenation and oxidation of the acid with ozone are described herein.

As it was reported in the previous paper, the main component of limonene monozonide is 1,2-monozonide, following a small quantity of 8,9-monozonide. Limonene diozonide was gradually offered when limonene was ozonized for a longer time. It was found that the yields of β-isopropenyl-δ-acetyl-*n*-valeric aldehyde (IX) and acid (VI) degradated from limonen monozonide, gradually decreased when much volume of ozone was absorbed, and also that the yield of some unknown water-soluble substance which gave semicarbazone (m.p. 196–197°), gradually increased.

On the other hand, esterification of the degradation product of limonene diozonide with methyl alcohol which contained 3% hydrochloric acid, gave the same methyl ester as described in the literature. The semicarbazone of the methyl ester (XIII) melted at 172–173°.

Harries and Adam presumed that diacetylvaleric acid obtained by the oxidation of limonene diozonide with chromic oxide, gave cyclic acid (IV or IV). In order to prove the structure of the cyclic acid, esterification of the methyl ester (XIII), hydrogenation of the ethyl ester (XIV) and oxidation of (XIV) with ozone were performed.

Saponification of (XIII), gave monoketonic acid as well as the water-soluble substance which was described above. Infrared spectra of the methyl and ethyl esters indicated the presence of the conjugated double bond with the ketonic group. Ethyl ester of the saturated acid (XV), was obtained by the catalytic hydrogenation of (XIV). If the structure of the unsaturated acid is (IV), the ethyl ester of the saturated acid must be 2-oxo-4-methyl-cyclohexane-acetic acid ethyl ester (XV), and the melting point of the semicarbazone must be 116°, as described in the literature. However the melting point was 164–165°. Thereupon the structure of (IV′) was suggested as the degradated acid of limonene diozonide.

Oxidation of ethyl ester of the unsaturated acid (XIV), with ozone gave diester of ketodicarbonic acid (XVI). Saponification value and analysis indicated that of dicarbonic acid diethyl ester, but its structure could not be proved with certainty.

These cyclic acid and esters were also given by the oxidation of (IX) and (VI), described in the previous paper as degradation products of limonene monozonide, with ozone. Harries' presumption which suggested the presence of the cyclic acid (IV or IV′) as the degradation product of limonene

* This report was presented in part at the Annual Meeting of the Agric. Chem. Soc. Japan, Univ. of Tokyo, March 30, (1955).

** Present address; Kagoshima Regional Bureau, Japan Monopoly Corporation, Kagoshima.

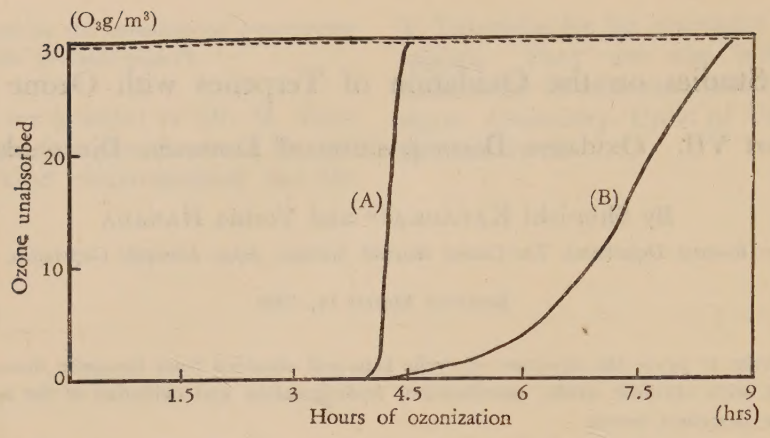


FIG. 1 Absorption Curves of Ozone for 30 g of Limonene (B), Pinene, Terpineol and *p*-Menthene-1 (A).

diozonide was found to be in agreement with our results. But more detail investigation must be made to prove the structure of the cyclic acid.

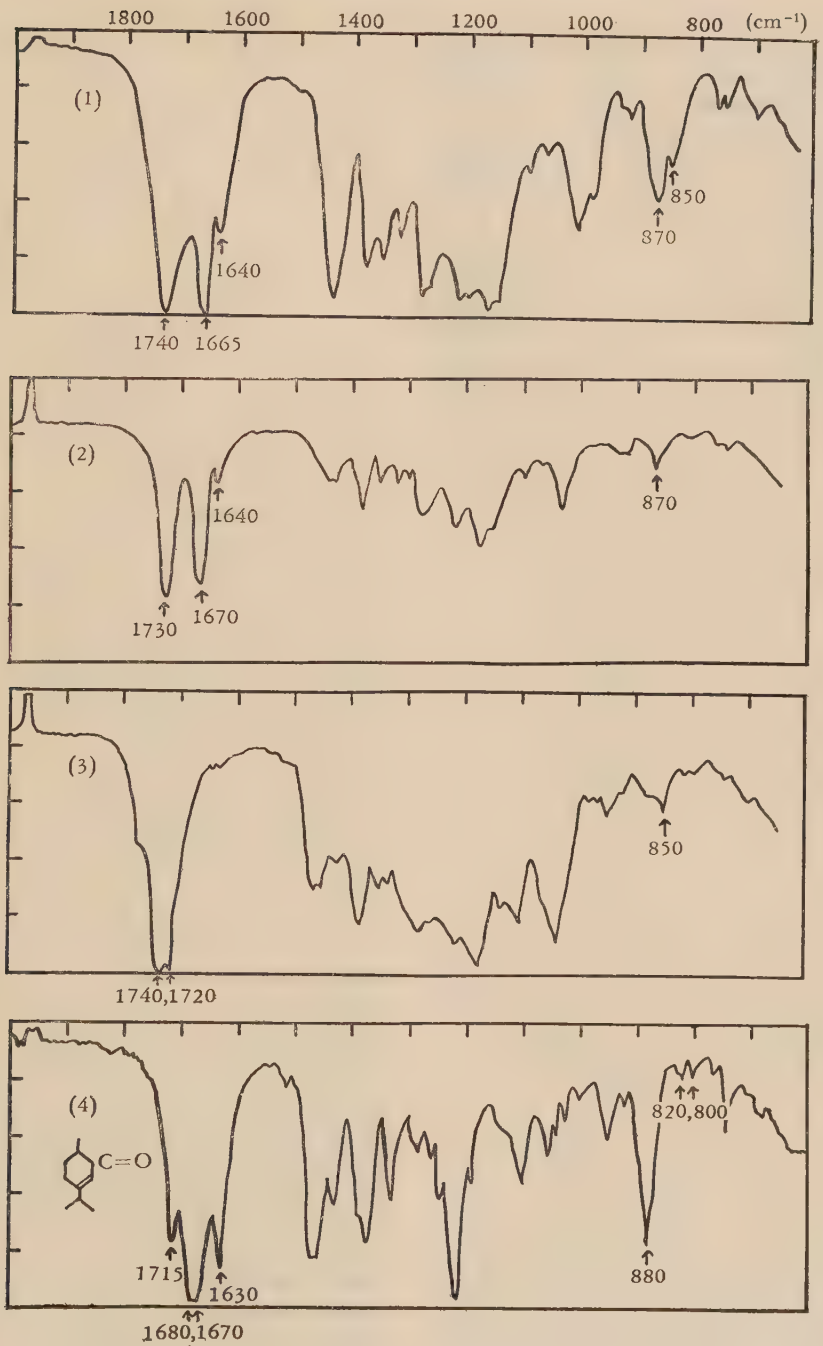
RESULT AND DISCUSSION

Curves in Fig. 1 show the absorption rates of ozone to limonene (B), pinene, terpineol and *p*-menthene-1 (A). As it was reported by Brus and co-workers[1] in the case of ozonization of limonene, one double bond adds ozone rapidly while the other adds it slowly. The time, calculated to saturate one double bond of limonene is three hours under the conditions of this report, but in our experiment nine hours were necessary to saturate two double bonds. White deep smoke yielded on ozonization, but vanished four and a half hours afterwards.

In the previous paper, ozonization for three hours (a) was reported. Ozonization for four and a half hours (b) and nine hours (c) were performed here. Yields of degradation products decreased to 9.8 g (b) and 2.6 g (c) in the neutral part as compared with 11.8 g (a). The yield of the acidic part also decreased to 4.0 g (b) and 1.7 g (c) as compared with 5.2 g (a). It was observed that degradation products of (a) and (b) were *β*-isopropenyl-*δ*-acetyl-*n*-valeric aldehyde (IX) and acid (VI), and that the product of (c) was an unknown water-soluble component degradated from limonene diozonide. The semicarbazone of the unknown acid melted at 196–197°. Analysis of semicarbazone agreed with that of the cyclic acid "A" (IV or IV′) suggested by Harries.

Harries esterified the degradation product, without separating the water-soluble acid, and obtained acid methyl ester (semicarbazone m.p. 172–173°). It was found that the same methyl ester (XIII) with Harries' methyl ester was obtained by the same procedure, and that saponification of the ester gave the water-soluble acid described above. It was presumed that the ester had a double bond, as it absorbed bromine. The ethyl ester (XIV) was also obtained. Infrared spectra of these esters are shown in Fig. 2 (1) (2). Wave numbers of 1740 and 1730 cm^{-1} indicated the presence of RCOOR′. It was presumed that 1655 and 1670 cm^{-1} indicated the presence of the C=O group conjugated with a double bond, and that 1640 and 870 cm^{-1} indicated the presence of a double bond of the $R_1R_2C=CHR_3$ type, conjugated

1) G. Brus and G. Peyresblanques, *Compt. rend.*, **190**, I, 501 (1930).



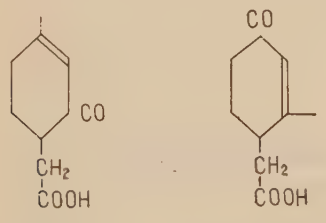
(1) Cyclic Acid "A" Methyl Ester (XIII) (2) Cyclic Acid "A" Ethyl Ester (XIV)
(3) Hydrogenated "A" Ethyl Ester (XV) (4) Carvenone

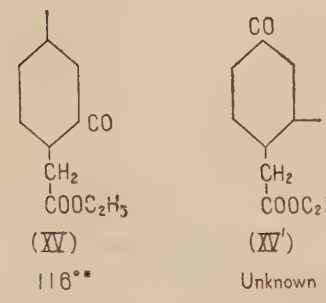
FIG. 2. Infrared Spectra.

with the ketonic group.

In order to elucidate the difference of wave number by conjugation, infrared spectrum of carvenone, obtained from limonene-1,2-glycol by the action of sulfuric acid was measured. This glycol has been obtained from limonene by the oxidation of benzoyl peroxide by Kitajima and Furukawa[2].

As shown in Fig. 2 (4), absorption wave numbers of C=O and C=C differ each other to 1670 and 1680, 880 cm^{-1}, by the conjugation. Therefore, the conjugation of C=C with C=O in the cyclic acid degradated from limonene diozonide was agreed. It is suggested that the wave number of 1715 cm^{-1} in Fig. 2 (4) is the absorption of some other C=O contained as impurity in the carvenone



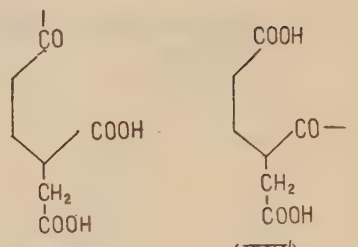


* m.p. of the semicarbazone from the literature.

FIG. 3

fraction, but this will be described in the preceeding paper.

If the structure of the unsaturated acid is



* m.p. from the literature.

FIG. 4.

(2-oxo-4-methyl-cyclohexene-3)-acetic acid (IV) as suggested by Harries, the structure of the saturated acid ethyl ester must be 2-oxo-4-methyl-cyclohexane-acetic acid ethyl ester. The semicarbazone of the ester obtined by Kötz[3] in the course of menthone synthesis, melted at 116°. Hydrogenation of cyclic acid "A" ethyl ester (XIV) gave ethyl ester of the saturated acid, but the melting point of the semicarbazone was 164–165°. Therefore, cyclic acid "A" must assume another structure (IV).

But however showings of the infrared spectrum of (XV) (Fig. 2 (3)), revealed that wave numbers of 1640 and 870 cm^{-1} vanished by the hydrogenation of the double bond, and the wave number of 1670 cm^{-1} which differed by the conjugation, was changed to 1720 cm^{-1} which was the absorption of the saturated six-membered ring ketone.

Oxidation of (XIV) with ozone and esterification gave diester (XVI). (XVI) gave semicarbazone (m.p. 78–80°). According to its saponification value and analysis, (XVI) will be monoketocarbonic acid diethyl ester. Saponification of (XVI) gave crystal (m.p. 60–80°), but this crystal could not be purified. This acid should be β-carboxy-δ-acetyl-*n*-valeric acid (XVII) (m.p. 119–120°) or β-acetyl-adipic acid (XVII') (m.p. 102°), but could not be accurately determined.

Cyclic acid "A" and the ester were also

2) M. Kitajima and K. Furukawa et al., *Shōnō-Kenkyū (Rep. of Camphor Research Dep., The Central Research Inst., J.M.C.)* **4**, 17 (1952).

3) A. Kötz and A. Bieber et al., *Ann.* **350**, 243 (1906).

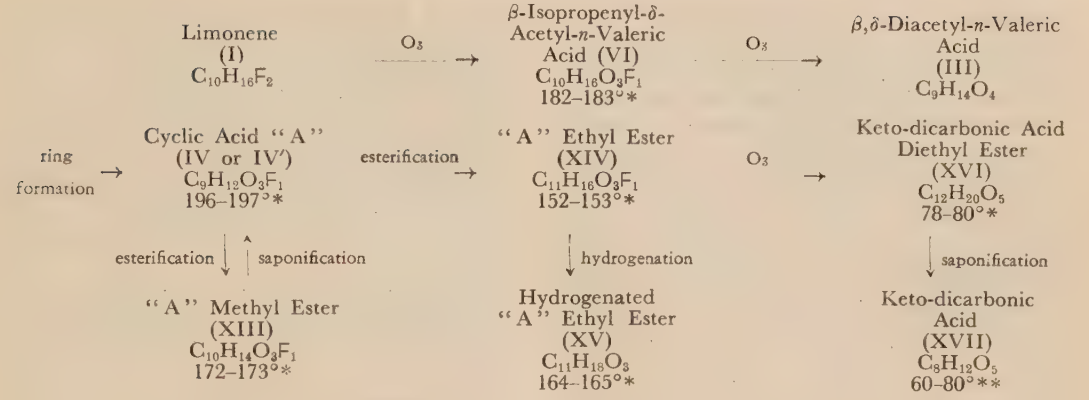


FIG. 5.

given by the oxidation of (IX) and (VI) with ozone. The scheme of the above reaction is considered as shown in Fig. 5.

Spencer[4], considered that limonene was ozonated to diozonide in the vapor phase only, and that could not be ozonated in the liquid phase. But the production of limonene diozonide in the liquid phase was found to agree with our results. And the production of the cyclic keto-acid, possessing the conjugated double bond with the ketonic group, agreed with the degradation product of limonene diozonide.

EXPERIMENTAL

1. Ozonization. Limonene (30 g) (b.p. 79.5°/30mm, d_4^{25} 0.8397, n_D^{25} 1.4699, $[\alpha]_D^{17}$+119.03°) in glacial acetic acid (450 g) was ozonated by an ozone stream (oxygene pass 120 l/hr., concentration of ozone 29.55 g/m^3, yield of ozone 3.546 g/hr.). Analyses of the unabsorbed ozone were performed by the general method[5], in which the colorized time of the potassium iodide solution containing quantitative potassium thiosulfate solution by the pass of ozone stream was measured.

Ozonization was performed for three hours (a), four and a half hours (b) and nine hours (c). The solution was poured into a three-necked flask, cooled under 20° and added acetic acid (1 l) solution, containing chromic oxide (14 g) under stirring in the case of (a) and (b). In the case of (c), acetic acid (2 l) solution containing chromic oxide (28 g) was added. After two days, the acetic acid was distilled under a reduced pressure, at about 50°. The extraction of the distilled residue of the acetic acid solution with ether was performed several times, and the ether fraction was washed with aqueous solution sufficiently. After the above procedure, this ether fraction were dealt with the same procedure by sodium hydroxide solution as in the previous paper.

(a) Neutral part 11.8 g (d_4^{25} 1.0227, n_D^{25} 1.4790): Acidic part 5.2 g (d_4^{25} 1.0566, n_D^{25} 1.4685), (b) N.P. 9.8 g (d_4^{25} 1.0383, n_D^{25} 1.4832): A.P. 4.0 g (d_4^{25} 1.0857, n_D^{25} 1.4871), (c) N.P. 2.6 g (d_4^{25} 1.1267, n_D^{25} 1.4869): A.P. 1.7 g (d_4^{25} 1.1086, n_D^{25} 1.4803) were obtained. Semicarbazones ((a) 0.3 g, (b) 0.3 g, (c) 0.1 g) were obtained from the acids (0.5 g) described above, and recrystallized from ethyl alcohol. These semicarbazones melted at (a) 182–183°, (b) 178–179°, (c) 195–197° (decomp.).

2. Cyclic Acid "A" (IV or IV'). The aqueous solution obtained in procedure (c) was concentrated under a reduced pressure below 50°, passing carbon dioxide. Viscous oil (15.5 g) was obtained. Action of semicarbazide hydrochloride and sodium acetate gave a large quantity of crystal two or seven days after. Semicarbazone (4.2 g) was obtained by the filtration and recrystallized from hot aqueous solution. This semicarbazone melted at 196–197° (decomp.). *Analysis* Found: N, 18.30. Calcd. for $C_{10}H_{15}O_3N_3F_1$: N, 18.68%.

4) C.C. Spencer and W.I. Weaver et al., *J. Org. Chem.*, **5**, 610 (1940).

5) H. Funahashi, *Rept. of Camphor Engineers Association*, **15** 66 (1950).

Besides this, ozonization of β-isopropenyl-δ-acetyl-*n*-valeric acid (10 g) obtained in the previous paper by the same procedure as described in **4**, gave the same water soluble acid (7.0 g), accompanying the original acid (3.3 g). This acid gave semicarbazone (1.26 g) (m.p. 196–197° (decomp.)). The melting point of the semicarbazone was not depressed by admixture with the semicarbazone obtained in procedure (c).

3. Methyl Ester of Acid "A" (XIII). Limonene diozonide, obtained from limonene (60 g) was oxidized by chromic oxide. After the distilling acetic acid, extraction with ether gave a red-brown viscous oil (58.7 g) involving acetic acid. Action of a fourfold volume of methyl alcohol invloving 3% hydrochloric acid for three days gave crude ester (21.5 g), after extraction with ether and washing with aqueous and 5% sodium carbonate solution. Fraction (a) (2.8 g) (b.p. 70–100°/2 mm, d_4^{25} 1.0357, n_D^{25} 1.4692), fraction (b) (10.0 g) (b.p. 100–136°/2 mm, d_4^{25} 1.0743, n_D^{25} 1.4732) and the residue (6.3 g) were obtained by distillation in vacuum. Redistillation of fraction (b) gave the methyl ester fraction (b.p. 105–115°/2 mm, d_4^{25} 1.0782, n^{25} 1.4744). The semicarbazone of this fraction melted at 172–173°, and the melting point was not depressed by admixture with the semicarbazone (m.p. 172–173°) obtained in the previous paper (part I). *Analysis* Found: N, 17.90. Calcd. for $C_{11}H_{17}O_3N_3F_1$: N, 17.57%. *Ester Value* Found: 324.71. Calcd. for $C_{10}H_{14}O_3$: 307.7.

Ester (6.2 g) was saponified on a steam bath for one hour with a 0.5 N alcoholic potassium hydroxide solution, neutralized with dilute sulfuric acid, and extracted with ether. The origial acid (1.2 g) (d_4^{25} 1.0671, n_D^{25} 1.4917) was obtained, and the semicarbazone (1.4 g) obtained from the aqueous solution melted at 195–196°. This melting point was not depressed by admixture with the semicarbazone obtained in **2**.

4. Oxidation of β-Isopropenyl-δ-Acetyl-*n*-Valeric Acid (VI) to Methyl Ester of Acid "A" with Ozone. Ozonization of limonene (90 g) by the same procedure described in the previous paper, gave N.P. (36.5 g) and A.P. (12.8 g). Acid (12.8 g) in glacial acetic acid (200 ml) was ozonized for one and a half hours, and decomposed with 30% hydrogene peroxide solution (10 ml) and ferrous sulfate (0.5 g) by heating on a steam bath for one hour, after standing for three days under room temperature. Esterification was performed by the same procedure as described above **3**. Redistillation of the obtained ester (6.6 g) gave the methyl ester fraction (b.p. 115°/2 mm, d_4^{25} 1.0370, n_D^{25} 1.4791). The melting point of the semicarbazone (0.4 g) obtained from the ester (0.5 g) was 172–173°, and was not depressed by admixture with the semicarbazone obtained in **3**. The phenylhydrazone (0.5 g) obtained from the ester (0.5 g) was a light yellow crystal and melted at 131–132°. Infrared spectrum of the above methyl ester is shown in Fig. 2 (1).

5. Ethyl Ester of Acid "A" (XIV). Ozonization of limonene (90 g) gave N.P. (40.2 g) and A.P. (10.4 g). Acid (10.4 g) was ozonized and esterified with ethyl alcohol involving 3% hydrochloric acid with the same procedure as described in **4**. Redistillation of the obtained ester (7.0 g) gave the colorless fraction (b.p. 120/2 mm, d_4^{25} 1.0570, n_D^{25} 1.4733). The semicarbazone (0.3 g) of the ethyl ester (0.5 g) melted at 152–153° after recrystallization three times from ethyl alcohol. *Analysis* Found: N, 16.36. Calcd. for $C_{12}H_{19}O_3N_3F_1$: N, 16.60%. Infrared spectrum of the ethyl ester is shown in Fig. 2 (2).

The ester fraction (18.0 g) was obtained from N.P. (40.2 g) by ozonization and esterification. Ethyl ester (5.8 g) (d_4^{25} 1.0551, n_D^{25} 1.4727, semicarbazone m.p. 152–153°) was obtained. Besides this fraction, keto-aldehyde fraction (3.0 g) (b.p. 70–115°/2 mm, d_4^{25} 1.0122, n_D^{25} 1.4609, semicarbazone m.p. 250–251° (decomp)) was also obtained.

6. Hydrogenation of Acid "A" Ethyl Ester. The ethyl ester absorbed bromine. (XIV) (7.4 g) was hydrogenated in an autoclave with methyl alcohol (10 ml) and Raney nickel (1 g) under 42 kg/cm². The depression of pressure was 12 kg/cm² (calcd. 11.5 kg/cm²), absorbing 1 mol. of hydrogen. After extraction with ether and washing with saturated sodium chloride solution, and removal of the solvent, afforded saturated acid ethyl ester (5.7 g). Distillation gave colorless oil (b.p. 110°/2 mm, d_4^{25} 1.0268, n_D^{25} 1.4548, semicarbazone m.p. 152–153°). *Analysis of semicarbazone* Found: N, 16.17: Calcd. for $C_{12}H_{21}O_3N_3$: N, 16.47%. The melting point was depressed to 134–136° by admixture with the semicarbazone of the original acid ethyl ester (m.p. 152–153°).

7. Oxidation of Acid "A" Ethyl Ester with Ozone. (XIV) (27.3 g) (b.b. 110–130°/3 mm, d_4^{25} 1.0580, n_D^{25} 1.4770) was obtained from limonene (210 g) by the same procedure as described in **5**. After three hours' ozonization, the same procedure gave

(XVI) (21.8 g) (b.p. 130–140°/1mm, d_4^{25} 1.0438, n_D^{25} 1.4442). The semicarbazone (0.7 g) obtained from the diester (1.0 g) melted at 78–80°, after recrystallization with 50% ethyl alcohol. *Analysis* Found: C, 52.16; H, 7.32; N, 14.12. Calcd. for $C_{13}H_{23}O_5N_3$: C, 51.83; H, 7.64; N, 13.95%. *Saponification Value* Found: 434.5. Calcd. for $C_{12}H_{20}O_5$: 459.0.

The diester (XVI) (19.5 g), was saponified with alcoholic potassium hydroxide. After distillation of alcohol, neutralization with dilute hydrochloric acid, concentration in carbon dioxide and extraction with absolute ethyl alcohol, gave a crystal (8.2 g) (m.p. 60–80°). But this crystal could not be purified.

The authors are grateful to Dr. M. Kitajima, Chief of the Camphor Research Department for his kind encouragement, Mr. K. Furukawa for his generous offering of the carvenone specimen and Mr. T. Takeshita for his assistance with infrared spectra. They are also indebted to the Laboratory of Microanalysis, Department of Agric. Chemistry, Univ. of Tokyo, for their microanalyses.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 236~242, 1956]

Studies on the Oxidation of Terpenes with Ozone

Part VIII. Oxidation of Hydrogenated Limonene with Ozone*

By Shin-ichi KATAOKA** and Yoshio HANADA

Camphor Research Department, The Central Research Institute, Japan Monopoly Corporation, Tokyo

Received May 10, 1956

Oxidation of hydrogenated limonene with ozone gave perfumery hexahydro-*p*-acetyltoluene which was in agreement with the synthetic specimen. It was concluded that *p*-menthene-1 was accompanied by a small quantity of 1-methyl-4-isopropenyl-cyclohexane as an intermediate in the hydrogenation of limonene. Additions of hydrogen, hydrochloric acid, oxygen and ozone to limonene are also discussed herein.

Smith and co-workers[1] reported that *p*-menthene-1 (I) was obtained only by the catalytic hydrogenation of limonene with a Raney nickel catalyst at room temperature, and acts as an intermediate in the hydrogenation of limonene to *p*-menthane. In the previous paper, (I) fractions obtained by the catalytic hydrogenation of limonene with copper chromite and Raney nickel catalysts, were oxidized with ozone. *β*-Isopropyl-*δ*-acetyl-*n*-valeric acid and two kinds of ketone (semicarbazone m.p. 208–209°, m.p. 161–162°) were obtained, but these ketones could not be accurately determined.

As the former ketone is considered to be the oxidation product of *p*-menthene-1, this will be reported in the preceding paper. In this report, infrared spectra of redistilled hydrogenated limonene fractions indicated the presence of 1-methyl-4-isopropenyl-cyclohexane (III) as the by-product of hydrogenation of limonene. Furthermore, the lower boiling ketone was found to agree with synthetic hexahydro-*p*-acetyltoluene (IV) obtained from isopulegol. It is concluded that (I) is accompanied by a small quantity of (III), as an intermediate in the hydrogenation of limonene.

Additions of hydrogen, hydrochloric acid, oxygen and ozone to the double bonds of limonene are also discussed in this report. It is concluded that one double bond of limonene is absorbed selectively, but another double bond is attacked as a secondary reaction, simultaneously. It was also concluded that hydrogen and hydrochloric acid are added in opposite directions of oxygen and ozone. Therefore, the added positions of these reagents will be presumed, in the case that the added position of hydrogen is known.

This is an interesting fact observed in the study of the oxidation of terpenes with ozone and the decomposition products of terpenes. This fact will be investigated more precisely in the preceding paper.

RESULTS AND DISCUSSION

Redistillation results of the menthene fraction obtained by the catalytic hydrogenation of limonene with Raney nickel catalyst at room temperature is shown in Table II. Infrared spectrum of every fraction was measured. As shown in Fig. 1 (1), 1, 2-double bonds of limonene indicated strong absorption in

* This report was presented in part at the 8th Annual Meeting of the Chem. Soc. Japan, Univ. of Tokyo, Apr. 3, (1955).

** Present address: Kagoshima Regional Bureau, Japan Monopoly Corporation, Kagoshima.

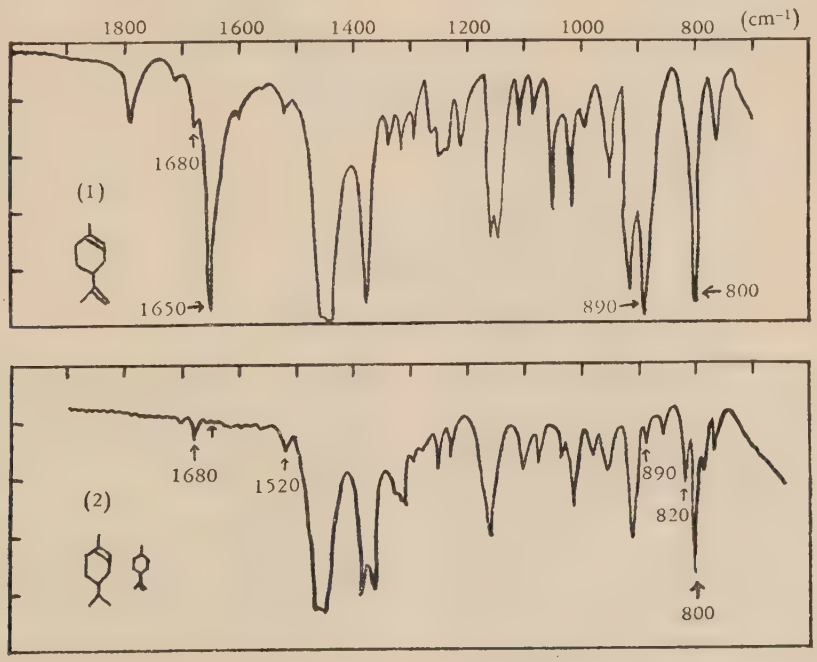
1) H.A. Smith, J.F. Fuzek, H.T. Meriwether, *J. Am. Chem Soc.*, **71**, 3765 (1949).

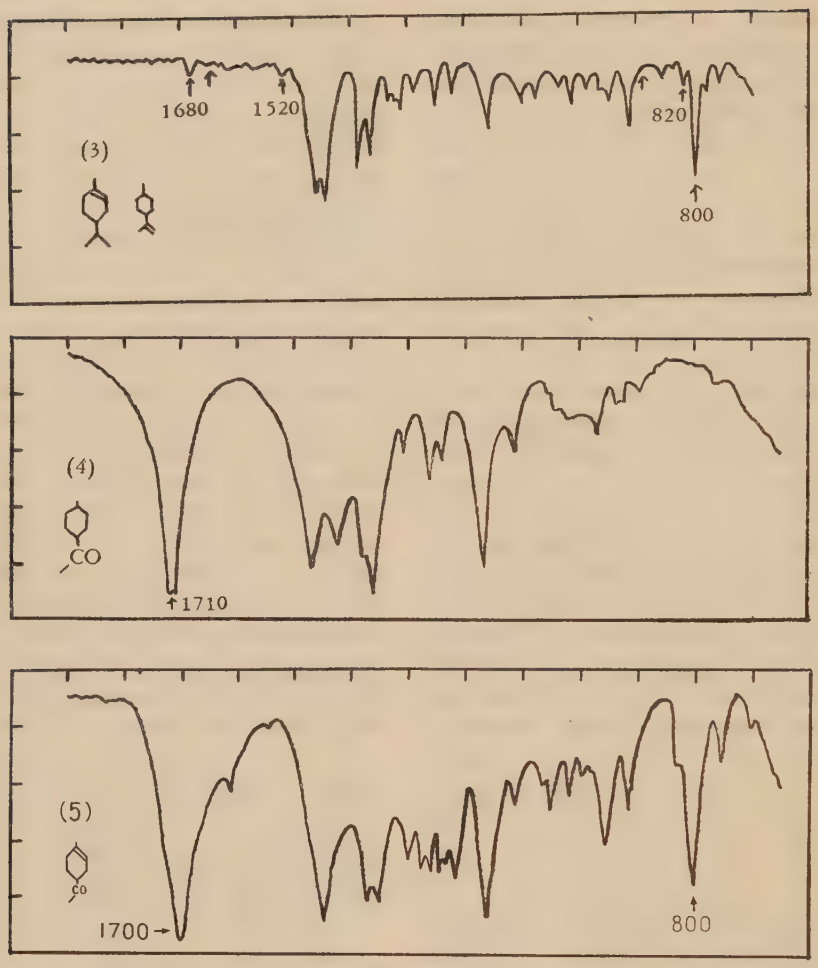
the wave number of 800 cm^{-1}, and weak absorption in 1680 cm^{-1}. These absorptions were observed in the spectra of *p*-menthene-1 (I), and are shown in Figs. (2), (3). The 8,9-double bond of limonene indicated strong absorptions in wave number of 890 and 1650 cm^{-1}. Among these absorptions, the wave number of 1650 cm^{-1} could not be found in menthene fractions (Figs. (2), (3)). A stronger absorption of 890 cm^{-1} could be slightly observed in Fig. (2), but could not be found in Fig. (3).

Slight absorption of the wave number of 890 cm^{-1} in Fig. (2), indicates the presence of 1-methyl-4-isopropenyl-cyclohexane (III) (b.p. 170°) which boils at a lower temperature than (I) (b.p. 177). As limonene (b.p. 177.6–178°) concentrates at a higher boiling fraction than fraction No. 7, the wave number of 890 cm^{-1} in fraction No. 3 is not the absorption number of limonene. Therefore, the presence of a small quantity of (III) was determined. It was concluded that fraction No. 7 was almost a pure (I) fraction.

Absorptions in 820 and 1520 cm^{-1} slightly observed in Fig. (2) and Fig. (3), were found to agree with the strongest absorption of *p*-cymene. It was concluded by infrared spectra that by-products in the hydrogenation of limonene to (I) were *p*-menthane and *p*-cymene. In part IV of this series, *p*-cymene was obtained in the catalytic hydrogenation of limonene with copper chromite catalyst under a high temperature, but it could not be obtained with Raney nickel catalyst under room temperature. *p*-Menthane was only obtained as the by-product in the later case.

The lower boiling fraction obtained by the redistillation of the neutral part, which was obtained by the oxidation of the above menthene fraction with ozone, gave semicarbazone (m.p. 161–162°). Analysis of semicarbazone agreed well with the theoretical value of hexahydro-*p*-acetyltoluene (IV). The regenerated oil from the semicarbazone indicated the infrared spectrum shown in Fig. 1 (4). In this spectrum strong absorption of ketone in the wave number of 1710 cm^{-1}





(1) Limonene
(2) Table II, No. 3 menthene fraction
(3) Table II, No. 7 menthene fraction
(4) Hexahydro-*p*-acetyltoluene
(5) Tetrahydro-*p*-acetyltoluene

FIG. 1. Infrared Spectra.

was observed, but the absorption of the double bond could not be observed. The infrared spectrum of tetrahydro-*p*-acetyltoluene obtained as the by-product by the oxidation of limonene monohydrochloride with ozone, was shown in Fig. 1 (5) as compared with Fig. (4). In this spectrum, the wave number of 800 cm^{-1} which indicated the presence of the $R_1R_2C=CHR_3$ type double bond, and the wave number of 1700 cm^{-1} indicating the presence of the ketonic group, were observed.

In order to confirm the production of (IV) with certainty, (IV) was synthesized from isopulegol by Semmler's procedure[2] shown in Fig. 2.

The semicarbazone of the obtained (IV) did not depress the melting point by admixture with the synthetic specimen. Physical properties of (IV) obtained in this report

2) F.W. Semmler, Ch. Rimpel, *Ber.* **39**, 2582 (1906).

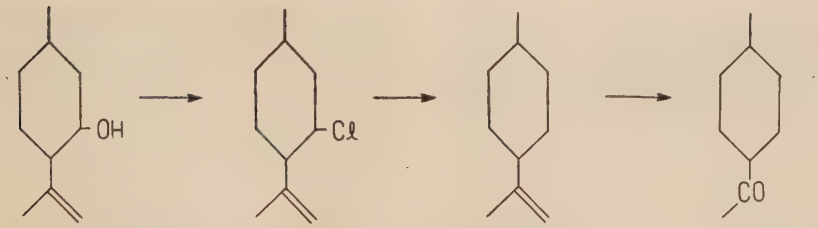


FIG. 2. Synthetic Method of Hexahydro-*p*-Acetyltoluene.

TABLE I
PHYSICAL PROPERTIES OF HEXAHYDRO-*P*-ACETYLTOLUENE

		b.p. °C/ 13 mm	d	n	semicarbazone
Findings of Semmler, Rimpel	Synthesis from citronellal	70~73	d_{20} 0.8982	n_D 1.4456	164~165°
Authors' findings*	Oxidation of the menthene fraction	70~71	d_4^{25} 0.8338	n_D^{25} 1.4322	161~162
Authors' findings	Synthesis from Isopulegol	70~81	d_4^{25} 0.9341	n_D^{25} 1.4450	161~162

* Regenerated oil from the semicarbazone.

and those in the literature are shown in Table I. Differences of specific gravities between the literature and this report are due to the impurities of both Semmler's and our synthetic specimen. The specific gravity of regenerated oil was shown in the oxidation product of the menthene fraction.

Our results were in agreement to the fact that the intermediate in the hydrogenation of limonene was (I) accompanied by a small quantity of (III), and that *p*-menthane was gradually produced. It was also concluded that the addition of hydrogen was mainly made to the 8,9-double bond, but a small quantity of hydrogen attacked the 1,2-double bond as a secondary reaction in the hydrogenation of limonene to *p*-menthane.

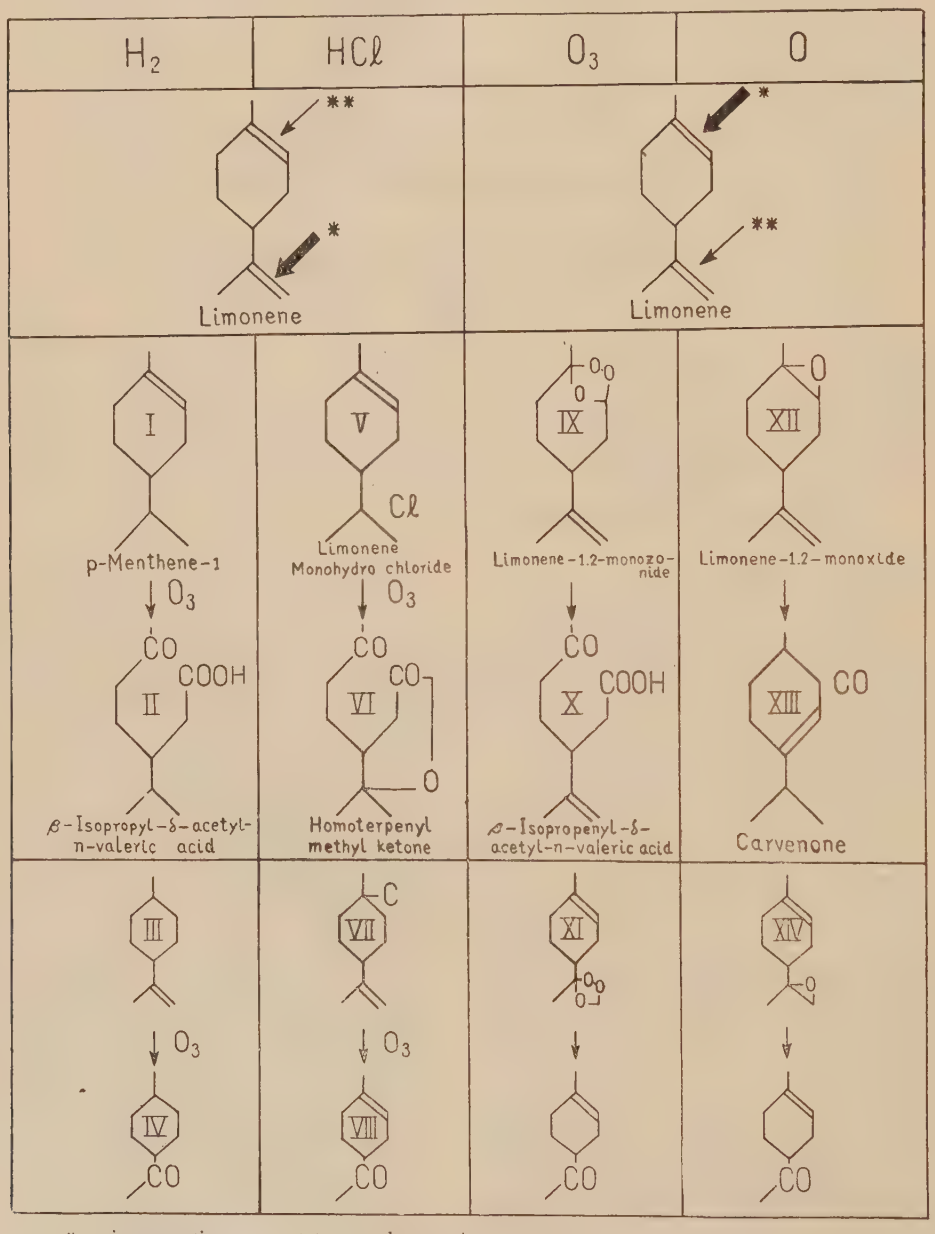
As reported in part VI of this series, the addition of ozone to limonene is mainly made to 1,2-double bond followed by secondary reaction attacking the 8,9-double bond. Ozone adds in the opposite direction of hydrogen as reported above.

Meerwein[3], obtained limonene-1,2-monoxide (XII) by the oxidation of limonene with benzoyl peroxide, and Sword[4], obtained limonene-1,2-glycol by the oxidation of limonene with hydrogen peroxide in an acetic acid solution. As it was reported in part VII of this series, another ketone was observed in the infrared spectrum of the carvenone fraction obtained by the decomposition of limonene-1,2-glycol with a dilute sulfuric acid. It was presumed that absorptions at 1715 and 800 cm^{-1} were that of tetrahydro-*p*-acetyltoluene (VIII). Therefore, oxygen attacks the 1,2-double bond mainly, but the 8,9-double bond will also be attacked as a secondary reaction, and this will produce limonene-8,9-monoxide (XIV). From this, it was concluded that the production of (VIII) was due to the decomposition of (XIV).

In part V of this series, homoterpenyl methyl ketone (VI), *p*-cymene and (VIII)

3) H, Meerwein, *J. prakt. Chem.*, **113**, II, 19 (1926).
4) Sword, *J. Chem. Soc*, 127, 1632 (1925).

were obtained by the oxidation of limonene monohydrochloride (V) with ozone, but the production of (VIII) could not be elucidated. Hydrochloric acid attacks the 8,9-double bond mainly, but the 1,2-double bond will also be attacked as a secondary reaction and will thus produce (VII). It was concluded that the production of (VIII) was due to the



* primary reaction. ** secondary reaction.

FIG. 3. Additional Scheme of Hydrogen, Hydrochloric Acid, Ozone and Oxygen to Limonene.

decomposition of (VII) ozonide accompanied with the removed reaction of hydrochloric acid.

As it was discussed above, additions of hydrogen, hydrochloric acid, oxygen and ozone were made to one double bond selectively, but another double bond was slightly attacked as a secondary reaction. Hydrogen and hydrochloric acid were added in opposite directions of oxygen and ozone. These relations are shown in Fig. 3.

The additional mechanism of ozone to terpenes possessing two kinds of double bond, can be presumed by these results, when the additional position of hydrogen is known. The additional mechanism of ozone to terpinolene will be reported in the preceding paper.

EXPERIMENTAL

1. Limonene. Tōhi oil (orange peel oil) was steam distilled, and vacuum distilled twice on a metallic sodium. Packed column: reflux ratio 1:20, number of theoreticl plate ca, 18, was used. Properties of the distilled limonene was as follows: b.p. 79.5°/30 mm, d_4^{25} 0.8397, n_D^{25} 1.4699, $[\alpha]_D^{17}$ +119.03°.

2. Hydrogenation. Limonene (200 g) in absolute ethyl alcohol (200 g) was added to Raney nickel (30 g) and hydrogenated in a 1,200 ml volume of autoclave at 132 kg/cm² pressure of hydrogen under stirring. About 1 mol. of hydrogen was absorbed for three hours. At this time, the reaction temperature rose from 17° to 24°. After the solution was filtered off from the recovered Raney nickel catalyst, it was extracted with ether. Ether solution was washed with saturated sodium chloride solution, and distilled. The properties of the distilled fraction (189.4 g) were as follows: b.p. 70–71°/20 mm, d_4^{25} 0.8191, n_D^{25} 1.4525, $[\alpha]_D^{12}$ +91.34°. Redistillation with the packed column described above gave the result shown in Table II (residue 2.9 g). Among the infrared spectra of every fraction, the spectra of fractions No. 3 and No. 7 are shown in Figs. 1 (2), (3).

TABLE II
FRACTIONAL DISTILLATION OF THE MENTHENE FRACTION OBTAINED BY THE HYDROGENATION OF LIMONENE

No.	b.p. °C/20 mm	Yield (g)	d_4^{25}	n_D^{25}	$[\alpha]_D^{13}$
1	65.5~69.5	23.1	0.8109	1.4448	+ 45.23°
2	69.5~70.5	23.2	0.8149	1.4501	+ 77.53
3	70.5	23.3	0.8158	1.4515	+ 85.32
4	70.5~71.0	23.6	0.8201	1.4530	+ 93.68
5	71.0	23.6	0.8217	1.4532	+101.01
6	71.0	23.5	0.8218	1.4549	+106.57
7	71.0	37.1	0.8224	1.4542	+111.05

3. Ozonization. Redistillation of No. 4—No. 7 (Table II) gave the menthene fraction (d_4^{25} 0.8212, n_D^{25} 1.4559, $[\alpha]_D^{14}$ +104.32°). This fraction (90 g) in a glacial acetic acid (450 g) was ozonated with an ozone stream as described in the previous paper. Heating of ozonide on a steam bath for one hour, distilling of acetic acid under reduced pressure and washing by 3% sodium hydroxide solution gave the Acidic Part (12.6 g) (d_4^{25} 1.0238, n_D^{25} 1.4592) and the Neutral Part (80.4 g). Besides these parts, the lower boiling fraction (5.1g) (d_4^{25} 0.8152, n_D^{25} 1.4432) was recovered from the distilled acetic acid solution.

A.P. was found to agree with β-isopropyl-δ-acetyl-*n*-valeric acid (II) obtained in part IV as compared with the authentic specimen. The lower boiling fraction was concluded as to be the mixture of *p*-menthane and *p*-cymene.

4. Hexahydro-*p*-Acetyltoluene (IV). Fractional distillation of N.P. (66.5 g) with Widmer's column is shown in Table III (residue 9.3 g). Semicarbazones of No. 1 and No. 2 were recrystallized twice, and melted at 161–162°. Analytical data agreed well with the theoretical value of hexahydro-*p*-acetyltoluene semicarbazone. *Analysis* Found: C, 61.14; H, 9.630; N, 21.47. Calcd for $C_{10}H_{19}ON_3$: C, 60.91; H, 9.645; N, 21.32%. Regenerated oil (1.7 g) (b.p. 70–71°/13mm, d_4^{25} 0.8338, n_D^{25} 1.4322) was obtained by steam distillation of the semicarbazone (2.6 g) with dilute sulfuric acid (20 ml). Infrared spectrum of the regenerated oil is shown in Fig. 1 (4).

TABLE III
FRACTIONAL DISTILLATION OF THE NEUTRAL PART OBTAINED BY THE OXIDATION OF MENTHENE FRACTION WITH OZONE

No.	b.p. °C/5 mm	Yield (g)	d_4^{25}	n_D^{25}
1	50~ 70	2.7	0.8737	1.4501
2	70~ 90	2.8	0.9126	1.4500
3	90~110	5.5	0.9419	1.4661
4	110~120	23.4	0.9710	1.4605
5	120~	22.8	1.0046	1.4627

5. Synthesis of Hexahydro-*p*-Acetyltoluene from Isopulegol. Isopulegol (50g) (b.p. 93–94°/14mm,

d_4^{25} 0.9176, n_D^{25} 1.4701) was dropped to phosphoric pentachloride (75 g) in hexane (200 ml) under cooling (5–10°). After two hours, the solution was washed by aqueous solution and crude isopulegol chloride (61.5 g) was obtained. Distillation gave isopulegol chloride (20.3 g) (b.p. 85–90°/12 mm, d_4^{25} 0.9433, n_D^{25} 1.4778).

Isopulegol chloride was poured into a flask of 500-ml volume eqquipped with Liebig's condenser, first added absolute alcohol (150 ml) and then added a metallic sodium (40 g) for two hours. Addition of ethyl alcohol (30 g), heating on a steam bath followed by steam distillation and distillation in vaccuum, gave 1-methyl-4-isopropenyl-cyclohexane (8.5 g) (b.p. 57–62°/14 mm, d_4^{25} 0.8114, n_D^{25} 1.4556).

This fraction in a glacial acetic acid (150 ml) was ozonated for one hour and a half, and then heated on a steam bath for three hours. Distillation of the obtained neutral part (4.9 g) gave hexahydro-*p*-acetyltoluene (2.1g) (b.p. 70–81°/13 mm, d_4^{25} 0.9341, n_D^{25} 1.4450). The semicarbazone melted at 161–162° after recrystallization thrice from ethyl alcohol, and the melting point was not depressed by admixture with the semicarbazone obtained in 4.

The authors are grateful to Dr. M. Kitajima, Chief of the Camphor Research Department for his kind encouragement, to Mr. T. Takeshita for his assistance with infrared spectra, and also to Mr. G. Indō, Takasago Perfumery Co. for his generous offering of isopulegol.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 243~251]

Studies on the Protease of *Pseudomonas*

Part I. The Production of the Enzyme under Various Cultural Conditions

By Kazuyuki MORIHARA

Research Laboratory of Shionogi & Co., Ltd.

Received May 1, 1956

The protease production of *Ps. myxogenes* sp. was found to be profoundly affected by some components of the cultural medium: Ca was indispensable for the protease production and a high concentration of glucose in the shaking culture was found to promote production remarkably, although they were not effective for growth of the organism, while Mg or organic nitrogen was effective for growth but suppressive for production of the enzyme.

Therefore, to obtain a high yield of protease, each of these influential components should be adjusted to their optimal concentration in the medium, so that the organism will be able to consume glucose in an adequately high concentration, in the presence of Ca.

INTRODUCTION

It is well known that enzyme production of a microorganism is remarkably affected by cultural conditions. Gale[1] summarized the factors on enzyme production as follows: (a) the chemical constitution of the cultural medium, (b) the physico-chemical conditions during growth, and (c) the age of cultivation.

In the present paper, experiments were carried out with *Ps. myxogenes* sp. which was isolated as an effective strain for the purification of 'lac' by Katagiri et al,[2] in order to ascertain whether various kinds of cultural conditions or media would affect its protease production.

EXPERIMENTAL METHODS

Ps. myxogenes sp. was cultured at 30°C on 200 ml of medium in 1-liter Fernbach-flasks (surface culture), or in 100 ml of medium in 500-ml Sakaguchi flasks kept on a shaker of 7 cm amplitude and 130 r.p.m. (shaking culture).

Protease activity was measured as follows: 1 ml of a diluted enzyme solution was added to 6 ml of 3% gelatin Palitzsch borate buffer solution of pH 7.0 containing 1% phenol in a test tube, and the mixture was held for 30 minutes at 35°C. From the reaction mixture, 5 ml of the solution was poured immediately into an Ostwald viscometer, and the time required for its outflow (E minutes) was measured at 30°C. This measurement should be made within 5 minutes. The same experiment was also carried out with a blank solution, and its time for outflow (C minutes) was measured. When the E/C was equal to 0.700, protease activity of the diluted enzyme solution was taken as 1 [u]/ml, provisionally. In an attempt to adjust the E/C to 0.700, the original enzyme solution employed i.e., the cultural filtrate* was diluted with water. The [u]/ml of the original enzyme solution should, therefore, be decided by the degree of dilution. By this method, the activity of crystalline trypsin (Mochida & Co., Ltd.) was measured, and it was found to be 120 [u]/mg.

Measurement of glucose in the cultural filtrate was carried out by the method of Bertrand, and the determination of NH_4Cl or $(NH_4)_2HPO_4$ was made by the semimicro Kjeldahl method in which the distillate of the cultural filtrate kept alkaline was absorbed in

1) E.F. Gale, *Bact. Rev.* **7**, 139 (1943).

2) H. Katagiri et al., *Bull. Inst. Chem. Res. Kyoto Univ.*, **18**, 41 (1949).

* The protease of *Ps. myxogenes* sp. was observed to be an extracellular enzyme.

0.01 N HCl.

The number of living cells in the cultural solution was calculated as follows: the cultural solution was diluted suitably with sterilized water and 1ml of the diluted solution was cultured in a Petri dish on bouillon agar for 3–5 days at 30°C. The number of the colonies thus formed on the plate, was determined, then the number of the living cells in the original solution was thereupon calculated from that of the colonies. The measurement was repeated five times in order to determine the average number of the living cells.

EXPERIMENTAL AND RESULTS

1. Effect of Ca and Mg. Natural media such as 5% soybean cake, bouillon, yeast extract and corn steep liquor were not effective for protease production of *Ps. myxogenes* sp., therefore, investigations were carried out to search for the most suitable synthetic medium (basal medium) for protease production by surface culture and it was found to be as follows: 1.0% glucose, 1.0% NH_4Cl, 1.0% Na_2HPO_4, 0.2% KH_2PO_4, 0.1% $CaCO_3$ and 0.05% $MgSO_4$.

The most favorable C-source was ascertained to be glucose or ethanol. Starch, dextrin, sucrose and various kinds of organic acids (excluding succinic acid) were found not to be useful as the C-source. As for the N-source, various kinds of proteins were found to give vigorous growth but unfavourably to protease production. Among the amino acids, glutamic and aspartic acids were found to be as effective as NH_4Cl, while the others not so effective. NH_4Cl was the most effective N-source among inorganic ammonium salts, such as $(NH_4)_2HPO_4$, NH_4NO_3 and $(NH_4)_2SO_4$.

Phosphate was indispensable for the culture of *Ps. myxogenes* sp., and it was considered that phosphate would contribute to the buffering action of the medium and also for some other unknown purposes.

It is of interest to find that Ca and Mg play an important part in protease production. To recognize the effect of Ca on protease production, $CaCO_3$, in the basal medium was replaced by other inorganic salts and protease production in such cultural medium comparatively determined. The same experiments were also performed to Mg. The results are shown in Table I.

Part A in Table I, shows that in media without $CaCO_3$, vigorous growth was observed in all cases excluding that with $CuSO_4$ but protease was not produced at all.

On the other hand, as shown in Part B in Table I, in all cultures with $CaCO_3$, protease was more or less produced even with scanty growth as in the case of $CaCO_3$ alone, but when $CaCO_3$ and $MgSO_4$ employed together, vigorous growth and high protease production was obtained. Either $MnSO_4$ or $Fe_2(SO_4)_3$ was effective for growth of the organism, but they were not so effective as $MgSO_4$ for its protease production.

Since the action of inorganic salts in the above

TABLE I

EFFECT OF INORGANIC SALTS ON THE PRODUCTION OF PROTEASE OF *PS. MYXOGENES* SP.

Part	Inorg. salt added (0.05%)	Growth 1 day	Growth 2 days	Growth 4 days	pH	Protease-activity [u]/ml
	—	+	##	##	5.6	0
A (containing 0.05% $MgSO_4$)	$BaCO_3$	±	+	##	〃	〃
	$ZnSO_4$	+	##	##	〃	〃
	$MnSO_4$	+	##	##	〃	〃
	$CuSO_4$	−	−	±	6.5	〃
	$FeSO_4$	##	##	###	5.6	〃
	$Fe_2(SO_4)_3$	##	##	###	〃	〃
	—	−	−	±	6.5	2.0
B (containing 0.1% $CaCO_3$)	$MgSO_4$	+	##	##	5.6	8.0
	$BaCO_3$	−	−	±	6.5	1.0
	$ZnSO_4$	−	±	+	5.6	2.5
	$MnSO_4$	+	##	##	5.6	4.0
	$CuSO_4$	−	−	±	6.5	0.5
	$FeSO_4$	±	+	##	5.6	0.2
	$Fe_2(SO_4)_3$	+	##	##	〃	1.5

The cultural medium contained 1.0% glucose, 1.0% NH_4Cl, 1.0% Na_2HPO_4, 0.2% KH_2PO_4 together with other salts shown in the table, and surface culture was conducted on 200 ml of the medium in a 1-l. Fernbach flask at 30°C for 4 days.

* Represents degree of growth according to thickness of the bacterial film: −) no; ±) slight; + ## ### ###) abundant

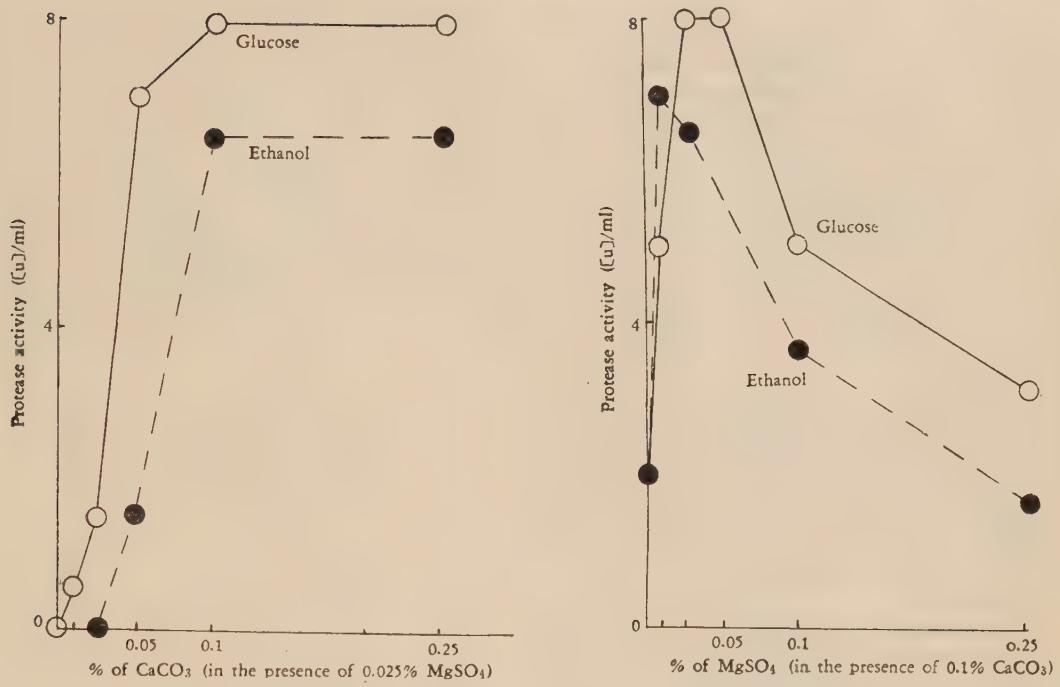


FIG. 1. Effect of Concentration of $CaCO_3$ or $MgSO_4$ on Protease Production of *Ps. myxogenes* sp. by Surface Culture in which Glucose ○——○ or Ethanol ●— —● was used as C Source

experiments was due to the cation, it can be said that Ca ion is essential for protease production and Mg ion is important for growth of *Ps. myxogenes* sp.

The optimum concentration of these salts in the basal medium were found to be 0.1% $CaCO_3$ and 0.01% $MgSO_4$ (in the ethanol medium) or 0.05% $MgSO_4$ (in the glucose medium), as shown in Fig. 1.

Very much the same effects of both Ca and Mg have also been pointed out even in media where ethanol was used as the C-source and protein, polypeptone or amino acid as the N-source. Therefore, it may be considered that the effect of Ca and Mg on protease production is generally significant for this organism.

As seen in Table I, $CaCO_3$ manifested no adjusting effect on pH in the cultures and Ca ion was found to have no activating action on protease itself, so, it can be said that Ca ion directly accelerated protease formation of the organism.

The fact that Ca and Mg are indispensable in the production of bacterial protease on a synthetic medium was demonstrated with *B. proteus vulgaris*, *B. subtilis*, or *B. Natto* by Merrill & Clark[3], Wilson[4], or Kanie and Morihara[5], respectively, but heretofore no demonstration of the above fact has yet been made on a natural medium.

2. Effect of the Concentration of Glucose. The protease production by shaking culture with the basal medium employed in surface culture was found to be very low. As for shaking culture, $(NH_4)_2HPO_4$ was observed to be more effective than NH_4Cl as the N-source, although even with the medium containing ammonium phosphate (i.e., 1.0% glucose, 1.0% $(NH_4)_2HPO_4$, 1.0% Na_2HPO_4, 0.2% KH_2PO_4, 0.1% $CaCO_3$, 0.05% $MgSO_4$) production of protease by shaking culture (1[u]/ml) was found to be lower than that by surface culture (8[u]/ml). The time required for complete consumption of glucose was shortened by shaking culture in one day, while it took more than 3 days was required by surface culture. However, in both cases it was found that the protease produc-

3) A.T. Merrill and W.M. Clark, *J. Bact.* **15**, 267 (1928).
4) E.D. Wilson, *ibid.* **20**, 41 (1930).
5) M. Kanie and K. Morihara, *Bull. Kagoshima Agr. College* **15**, 101 (1949).

tion reached the maximum at the time of consumption of glucose. It is already known that[6], some hydrolases are formed only when the C-source in a medium is almost completely consumed by microbes.

It seemed probable that by adopting the shaking culture method, glucose might be consumed in a much higher concentration by the organism within a relatively short period of time and consequently, protease might be produced in a much higher yield. On the other hand, by surface culture, a high concentration of glucose could not be consumed and almost no protease production was observed.

The following experiments were made in regard to this possibility:—

1) *Ps. myxogenes* sp. was cultured on a medium with various concentrations of glucose (0.5, 1.0 and 2.0%). It was found that enzymatic activity of the cultural solution enhanced (0.5, 1.0 and 10 [u]/ml) with the increase of the concentration of glucose, up to 3% in which glucose completely inhibited the growth owing to the increase of acidity of the cultural solution.

2) Therefore, the addition of $CaCO_3$ was required in order to neutralize the cultural solution containing more than 3% of glucose. The optimum concentrations of $CaCO_3$ were as follows: 0.2% for 3% glucose and 1.0% for 5% glucose media, thereby protease production further enhanced along with increase of the concentration of glucose (20 [u]/ml with 3% and 40 [u] with 5% glucose). Further addition of $CaCO_3$ over the optimum concentrations mentioned above caused inhibition of growth. That is to say, an excess of Ca inhibits growth of the bacterium.

3) In a medium containing over 7% glucose and 1.5% $CaCO_3$ necessary for the neutralization, growth was completely inhibited by these high concentrations. However, this inhibition could be overcome by the addition of more $MgSO_4$ to the medium. The data given in Fig. 2. indicates protease production in the medium containing such a high concentration of $MgSO_4$. Here again, Mg played its favorable role on growth of the organism. When corn steep liquor (C.S.L.) was added in place of the extra concentration of $MgSO_4$ to the medium containing 0.05% $MgSO_4$, 7% glucose and 1.5% $CaCO_3$, it was also found to be effective in promoting growth and in giving a remarkably large amount of protease (200 [u]/ml).

6) S. Yamaguchi and E. Matsusaki, *Symposia on Enz. Chem. (Japan)* **7**, 16 (1952): T. Asai, *J. Agr. Chem. Soc. (Japan)* **26**, 382 (1952): O. Tanabe and K. Tonomura, *ibid.* **28**, 229 (1954).

From the above experiments, the decisive factors of the protease production by shaking culture were ascertained to be as follows: (1) the concentration of glucose and (2) the appropriate concentrations of $CaCO_3$ and C.S.L. A series of experiments were carried out in order to find the optimum concentrations of $CaCO_3$ and C.S.L. corresponding to that of glucose in the medium: the results are shown in Fig. 3, where (A) represents results with 2% glucose, (B) with 4% glucose and (C) with 7% and 10% glucose. The most effective concentrations of $CaCO_3$ and C.S.L. to various concentrations of glucose for

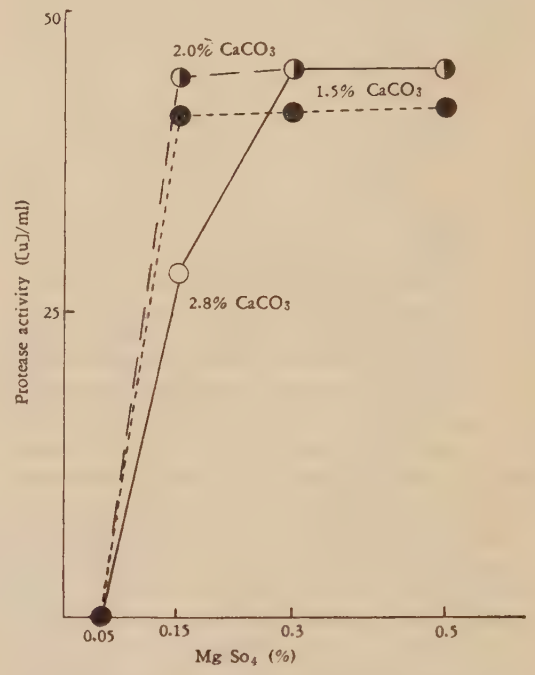
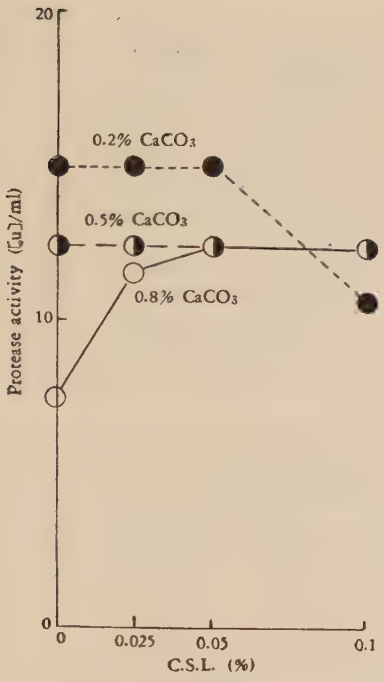


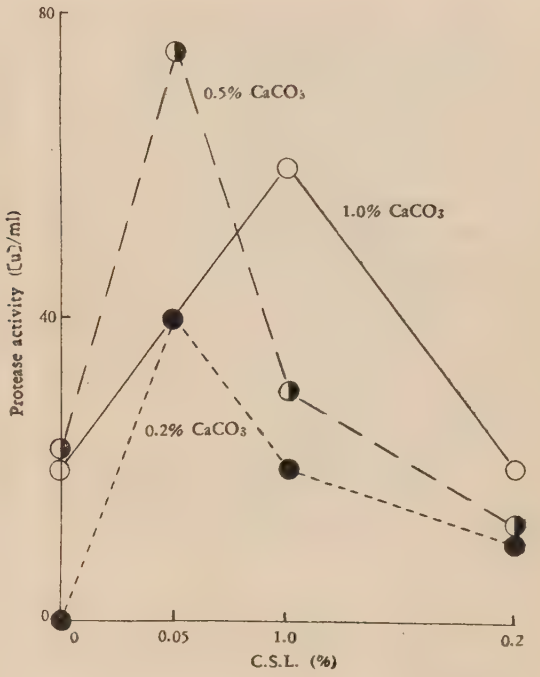
FIG. 2. Effect of the Concentration of $MgSO_4$ by Shaking Culture on the Medium Containing 7% Glucose, 1.0% $(NH_4)_2HPO_4$, 1.0% Na_2HPO_4, 0.2% KH_2PO_4 and Various Concentrations of $CaCO_3$, for Protease Production of *Ps. myxogenes* sp.

● ····● ···· ····· 1.5% $CaCO_3$
◑——◑ ····· 2% $CaCO_3$
○——○ ···· ····· 2.8% $CaCO_3$

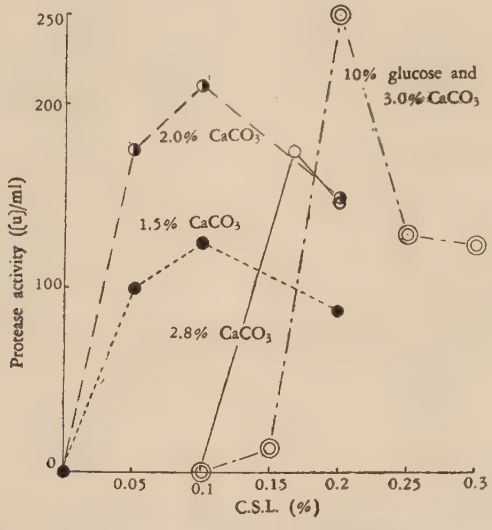
protease production were found to be as follows: 0.2% $CaCO_3$ for 2% glucose (A), 0.5% $CaCO_3$ and 0.05% C.S.L. for 4% glucose (B), 2% $CaCO_3$ and 0.1% C.S.L. for 7% glucose, and 3% $CaCO_3$ and 0.2% C.S.L. for 10% glucose (C). The maximum production of protease in these cases were 15 [u],



A) 2.0% glucose (Cultured for 3 days)
●·····● 0.2% $CaCO_3$, ◑– – –◑ 0.5% ○——○ 0.3%



B) 4.0% glucose (Cultured for 3 days)
●·····● 0.2% $CaCO_3$, ◑– – –◑ 0.5% ○——○ 1.0%



C) 7.0% glucose (Cultured for 4 days) and 10% glucose (Cultured for 5 days)
● ···· ● 1.5% $CaCO_3$ and 7% glucose ◑– – –◑ 2.0% $CaCO_3$ and 7% glucose
○——○ 2.8% $CaCO_3$ and 7% glucose ◎ –•–◎ 3% $CaCO_3$ and 10% glucose

FIG. 3. Effect of the Concentrations of Glucose, C.S.L. and $CaCO_3$ by Shaking Culture Containing 1.0% $(NH_4)_2HPO_4$, 1.0% Na_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4$ on the Protease Production of *Ps. myxogenes* sp.

75 [u], 200 [u] and 240 [u] per milliliter, respectively. Namely, the production of protease by shaking culture remarkably enhanced with increase of the concentration of glucose in the medium. The maximum value (240 [u]) obtained by shaking culture is about 30 times as high as that of surface culture (8 [u]).

Moreover, experiments were made as to decide whether the other organic N-sources were as effective as C.S.L. on the protease production in the medium containing a high concentration of glucose and $CaCO_3$. This was verified as: i.g., 250 [u] by the addition of 0.2% yeast extract, 200 [u] by the addition of 0.2% peptone, 150 [u] by the addition of 0.2% glutamic acid, and 130 [u] by the addition of 0.2% aspartic acid respectively, as a substitute for C.S.L. to the medium containing 7% glucose.

It is of interest to note here that an excess of C.S.L. remarkably lowers protease production, therefore the concentration of C.S.L. in a medium necessiated for production must be the minimum amount required for bacterial growth.

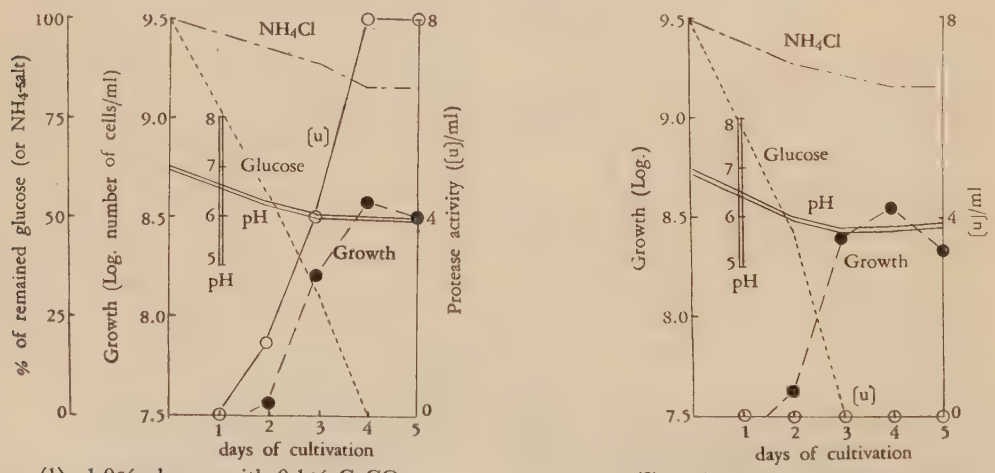
Thus, the author next investigated the relation between protease production and growth or transition of each component of the medium under various cultural conditions.

In regard to surface culture, the basal media with or without $CaCO_3$ was used (A). For shaking culture, a media containing various concentrations of glucose, i.e., 1, 2, 4 and 7% and also containing $CaCO_3$ and C.S.L. in each optimal concentration, which thereby gave the highest protease production (as shown in Fig. 3) were used (B), and the medium containing 7% glucose and $MgSO_4$ or C.S.L. in a higher concentration than the optimum, which thereby gave a low protease production was used for comparative study (C). One ml of the cell suspension containing 10^8 cells/ml was inoculated into each cultural solution, and in each culture various observations were made during cultivation; the results are shown in Fig. 4.

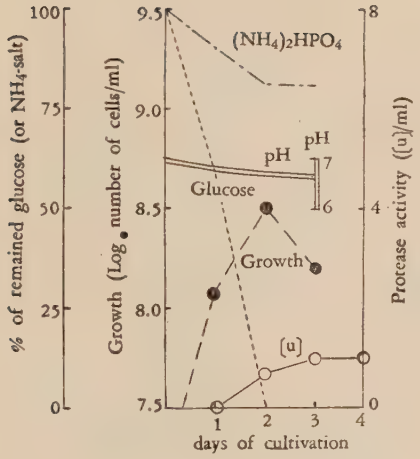
Fig. 4 indicates that, at the time when glucose was completely consumed and NH_4-salt was utilized to its highest degree in the medium, growth reaches its maximum, and in all cases, it was observed that protease production is the highest at the same time or a little later.

By surface culture (A), consumption of glucose was always observed and the rate of utilization of NH_4-salt and bacterial growth on the cultural medium were found to be almost equal in both cases, being independent of the presence of $CaCO_3$. In the case of shaking culture (B), the higher the concentration of glucose, the more the NH_4-salt was utilized and more abundant growth was observed. However, the ratio of ascension of the protease production to the increase of the concentration of glucose in the medium was found to be remarkably greater than the ratio to the increase of growth. It may, therefore, be said that the increase of the enzyme production can not be merely ascribed to the bacterial growth. The following results may clear up that point.

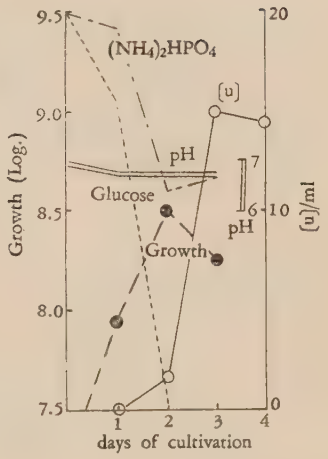
Protease production was found to be lower (20–47



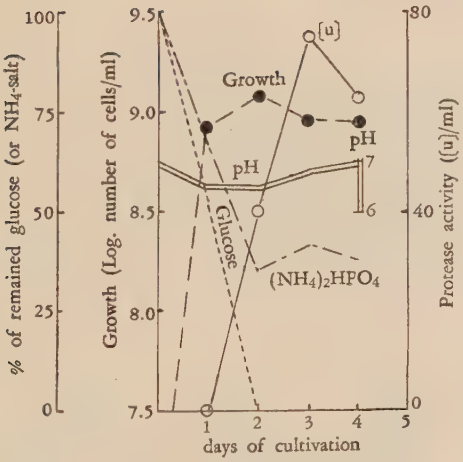
(A) Effect of $CaCO_3$ on surface cultures containing 0.1% NH_4Cl, 1.0% Na_2HPO_4, 0.2% KH_2PO_4 and 0.05% $MgSO_4$.



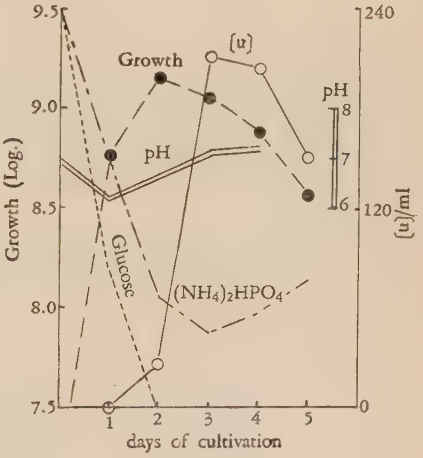
(1) 1.0% glucose and 0.05% $CaCO_3$



(2) 2% glucose and 0.2% $CaCO_3$

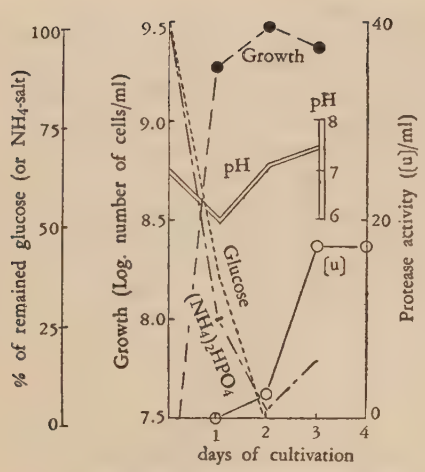


(3) 4% glucose, 0.5% $CaCO_3$ and 0.05% C.S.L.

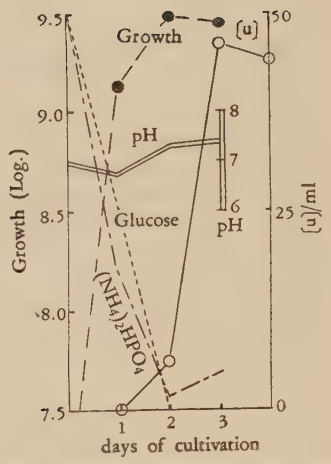


(4) 7% glucose, 2% $CaCO_3$ and 0.125% C.S.L.

(B) Effect of $CaCO_3$ or C.S.L. on shaking cultures containing various concentration of glucose, 1.0% $(NH_4)_2HPO_4$, 1.0% Na_2HPO_4, 0.2% KH_2PO_4 and 0.05% $MgSO_4$.



(1) 7% glucose, 2% $CaCO_3$ and 0.7% C.S.L.



(2) 7% glucose, 2% $CaCO_3$ and 0.5% $MgSO_4$.

(C) Effect of high concentration of $MgSO_4$ or C.S.L. on shaking culture containing 7% glucose and the mineral matters mentioned above.

FIG. 4. Relation between Protease Production and Bacterial Growth or Fluctuation of the Components in Various Cultural Solutions of *Ps. myxogenes* sp.

○——○ ..... Protease activity ([u]/ml)
●– – –● ..... bacterial growth
.... glucose remained
– • – • – ..... NH_4-salt remained
..... pH

[u]/ml) in the medium containing 7% glucose supplemented with an excess amount of C.S.L. or $MgSO_4$, by shaking culture (C) than the corresponding culture in series (B) (210 [u]/ml), in spite of the higher utilization of NH_4-salt and about twofold increase of bacterial growth.

In the medium containing 1% glucose, protease production by surface culture was observed to be higher than that by shaking culture, but no difference either in the utilization of NH_4-salt or in the bacterial growth was observed ((A) and (B)).

In order to obtain a more distinct understanding of the results shown in Fig. 4, protease activity per cell was calculated as shown in Table II.

From Table II it can be seen that protease activity per cells (10^8 cells) differs remarkably according to cultural conditions. Therefore, it may be concluded that the protease production of this organism is con-

TABLE II

RATIO OF PROTEASE PRODUCTION TO BACTERIAL CELLS UNDER VARIOUS CULTURAL CONDITIONS

Part	Glucose concentration of the medium (%)	Period of cultivation (days)	NH_4-salt consumed (%)	Protease-activity ([u]/ml)	Growth (Number of cells/ml)	Ratio of protease activity to growth ([u]/10^8 cells)
(A)	1.0 (with Ca)	4	19	8	$10^{8.63}$	1.9
	1.0 (without Ca)	4	19	0	$10^{8.54}$	0
(B)	1.0	3	20	1	$10^{8.50}$	0.3
	2.0	3	45	15	$10^{8.95}$	1.4
	4.0	3	65	75	$10^{9.10}$	5.2
	7.0	3	83	210	$10^{9.17}$	12.4
(C)	7.0 ($MgSO_4$)	3	95	47	$10^{9.49}$	1.22
	〃 (C.S.L.)	3	97	18	$10^{9.49}$	0.51

Parts (A), (B) and (C) were all calculated from the experimental results shown in Fig. 4.

siderably affected by cultural conditions but not necessarily by growth.

SUMMARY

The protease production of *Ps. myxogenes* sp. under various cultural conditions is studied.

Ca is indispensable for protease production and Mg is important for growth.

Aeration is necessiated for promotion of protease production. And the production by shaking culture is remarkably accerelated along with the increasing concentration of glucose in the medium containing other components of optimal concentration, that is, 1 〔u〕 per milliliter protease is produced with 1.0%, 15 〔u〕 with 2.0%, 75 〔u〕 with 4.0%, 210 〔u〕 with 7.0% and 240 〔u〕 with 10% glucose media. Maximum activity by shaking culture is found to be about 30 times to that by surface culture.

It is observed that enzyme production is not always attributable to growth, but depends on the productivity of the cell on various conditions.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. and also to Mr. E. Masuo of this Laboratory, for their constant guidance and encouragement in this work. He also wishes to thank Dr. K. Takeda, Director of this Laboratory, for approval of this publication.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 252~256, 1956]

Studies on the Proteolytic Enzymes of Black *Aspergilli*

Part I. Investigation of Strains Producing Superior Proteinase Yields in Black *Aspergillus* and the Crystallization of Proteolytic Enzyme from *Aspergillus Saitoi*

By Fumihiko Yoshida

Noda Industrial and Scientific Research Laboratory

Received July 9, 1956

A new proteolytic enzyme was obtained from Black *Aspergillus*. The pH, optimal for milk casein digestion by this enzyme lies in the region of 2.5–3.0. The crystallization of this enzyme from *Aspergillus Saitoi* is described here.

The properties of microbial proteinase have been reported by several authors such as Ayers[1], Dion[2], Crewther[3], Elliot[4], Dworschack[5] and others.

As a result of examining the proteolytic enzyme of molds such as *Asp. Saitoi*, *Asp. Usamii*, *Asp. awamori*, and other Black *Aspergillus*[6,7], the author found the proteolytic enzyme to have characteristic properties—the optimal pH for milk casein digestion was in the range of 2.5 to 3.5 and also shows a strong resistance to treatment under the following conditions; pH 2.0, 35°C 1 hr.—this differing from that of other *Aspergillus* species.

The strains belonging to Black *Aspergillus* contain proteolytic enzymes just as strong as *Asp. oryzae* and *Asp. sojae*. The result of this study as well as a discussion on the proteinase system and the crystallization procedure of this enzyme are presented in this paper.

1) G.B. Ayers and W.C. Tobie, *J. Bact.* **45**, 18 (1943).
2) W.M. Dion, *Can. J. Research.* C **28**, 577 (1950).
3) W.C. Crewther and F.G. Lennox, *Aust. J. Biol. Sci.* **6**, 410 (1953).
4) S.D. Elliot, *J. Exptl. Med.* **92**, 201 (1950).
5) R.G. Dworschack, H.J. Koepsell and A.A. Lagoda, *Arch. Biochem. Biophys.* **41**, 48 (1952).
6) K. Sakaguchi, H. Iizuka and S. Yamazaki, *J. Agr. Chem. Soc. Japan.* **24**, 138 (1951).
7) H. Iizuka, *J. Gen. Applied Microbiology.* **1**, 10 (1955).
8) F. Yoshida, *J. Agr. Chem. Soc. Japan.* **28**, 66 (1954).

MATERIALS AND METHODS

Production of Enzyme Solution. The mold strains were kindly supplied by the Sakaguchi Lab. Dept. of Agr. Chem. Univ. of Tokyo. The strains were grown for three days at 30°C on wheat bran and thereupon extracted with three folds of water, and the extract was assayed for the proteolytic enzymes.

Proteinase Assay. For investigations, the gelatin liquefaction was determined by the viscosimetry method of Matsuyama[9]. The milk casein and soybean-α-protein digestions were estimated by the measuring of trichloroacetic acid soluble nitrogen of the hydrolysate. The amino-nitrogen was estimated by the Van Slyke method.

In the purification procedure, proteinase activity was assayed by the modified Anson's method[10].

RESULTS AND DISCUSSION

(1) Investigation of Strains Producing Superior Proteinase Yields. Table I. gives a comparison of proteolytic enzyme activity of each species of Black *Aspergillus*.

The optimal pH for the hydrolysis of milk casein by each species always lies in the range of 2.5 to 3.0. Thus, it has hitherto been reported that the proteinase activity of Black

9) Y. Matsuyama and K. Shimura, *J. Agr. Chem. Soc. Japan.* **24**, 222 (1951).
10) M.L. Anson, *J. Gen. Physiol*, **22**, 79 (1938).

TABLE I

COMPARISON OF PROTEINASE ACTIVITY OF EACH SPECIES OF *BLACK ASPERGILLUS*

T.C.A. sol-N. mg.	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80
Asp. Usamii	1	1	1		1		1	
Asp. Saitoi	2	9	6	8	7	11	10	5
Asp. Inuii	7	6						
Asp. aureus	3	3	3	1				
Asp. Nakazawai	3	5	4					
Asp. awamori	22	35	11	3	6	7	6	1

TABLE II

STRAINS OF BLACK *ASPERGILLUS* PRODUCING SUPERIOR PROTEINASE YIELDS IN WHEAT-BRAN KOJI

Strains		Analytical data: Milk casein digestion (pH) 1.5	(pH) 2.5	(pH) 4.0	Soybean-α-protein digestion (pH) 1.5	(pH) 2.5	(pH) 4.5
Asp. Usamii	R–1031	45.31 mg	65.06 mg	24.86 mg	19.91 mg	37.29 mg	19.38 mg
Asp. Saitoi	K–2331A	40.95	66.06	31.50	29.40	42.00	27.30
〃	H– 611	30.24	62.47	39.90	20.43	39.39	29.92
〃	R–6711	25.20	65.10	44.20	26.67	53.97	34.65
〃	R–1216	49.56	63.00	44.10	19.95	44.10	31.50
〃	R–2941	27.30	71.40	38.90	29.40	56.07	33.60
〃	R–3221	32.55	65.10	37.80	27.82	38.35	33.60
〃	R–0122	27.30	71.40	43.05	33.60	48.82	37.80
〃	R–0521T	27.30	61.95	18.90	29.40	40.55	22.55
〃	R–0638	23.10	65.10	35.70	27.30	56.40	35.07
〃	R–0845	23.20	63.00	34.12	27.29	43.62	33.07
〃	R–1128	27.30	69.30	33.60	26.67	52.50	32.55
〃	R–1149	27.82	75.60	31.50	27.93	55.23	37.17
〃	R–3813	30.45	74.13	39.90	25.20	48.60	24.15
〃	R–4424	39.90	73.50	38.75	27.30	56.40	35.07
〃	R–5912	31.50	64.05	34.65	23.10	48.30	27.30
Asp. awamori	R–1618	19.95	65.86	25.20	27.30	46.20	31.50
〃	R–3523	33.60	71.40	45.19	27.30	49.35	33.60
〃	R–0736	19.95	61.63	23.45	22.05	44.10	32.55
		(5.0)	(6.5)	(7.5)	(5.0)	(6.5)	(7.5)
Asp. sojae	K.S.	18.18	53.63	57.03	31.08	38.05	37.80

Strains		Analytical data: NH_2-N liberated in soybean-α-protein digestion (pH) 1.5	(pH) 2.5	(pH) 4.0	gelatin liquefaction (pH) 1.5	(pH) 2.5	(pH) 4.5
Asp. Usamii	R–1031	4.047 mg	6.003 mg	3.726 mg	90.5 mg	86.4 mg	87.4 mg
Asp. Saitoi	K–2331A	6.184	7.833	4.306	86.3	85.3	85.2
〃	H– 611	4.511	6.152	3.691	89.4	86.4	85.4
〃	R–6711	3.353	6.244	3.945	88.1	86.3	86.3
〃	R–1216	3.449	5.796	4.988	87.8	86.3	85.0
〃	R–2941	4.748	6.606	3.715	90.3	85.7	85.3
〃	R–3221	3.256	5.293	3.923	85.9	82.3	81.6
〃	R–0122	4.532	7.003	3.985	89.8	87.7	86.6
〃	R–0521T	2.691	4.933	2.484	89.8	87.7	86.6
〃	R–0638	3.253	5.263	3.526	90.3	86.9	86.0
〃	R–0845	4.026	6.293	3.291	93.4	87.9	88.6
〃	R–1128	4.252	6.392	4.012	90.0	86.8	85.8
〃	R–1149	5.009	6.492	4.293	87.6	83.4	83.6
〃	R–3813	4.621	6.521	4.923	90.0	85.7	86.0
〃	R–4424	3.303	7.432	4.954	86.7	83.4	81.5
〃	R–5912	4.925	5.216	4.003	88.6	85.2	84.6
Asp. awamori	R–1618	4.616	6.084	5.245	—	—	—
〃	R–3523	5.657	10.910	8.812	89.6	87.5	86.7
〃	R–0736	5.245	9.441	7.152	91.8	88.2	84.3
		(5.0)	(6.5)	(7.5)	(5.0)	(6.5)	(7.5)
Asp. sojae	K.S.	4.970	6.490	3.526	75.9	75.2	75.6

TABLE III

EFFECT OF STARCH ADSORPTION IN VARIOUS CONCENTRATION OF ETHANOL

Preparation	PU*/mg Protein-N.	Yield	Dextrinogenic activity	Glucosidic-bond hydrolysed
Crude enzyme soln.	333	100%	10 min.	19%
10% (Prep. A)	452	64.9	45	13.6
20% (Prep. B)	544	67.7	45	11.2
30% (Prep. C)	616	74.5	65	9.6
40% (Prep. D)	79.3		100	7.6

* PU indicates milliequivalent of tyrosine, liberated per minute in the hydrolysis of 2% milk casein at 30°C.

TABLE IV

EFFECT OF AMMONIUM-SULPHATE FRACTIONATION OF PREP. D.

Preparation	PU/ Protein-N.	Yield	Dextrinogenic activity	Glucosidic-bond hydrolysed
Prep. D.	766	100%	100 min.	7.6%
0.4 satd. (Prep. E)	—	2.4	400	0
0.5 satd. (Prep. F)	687	12.4	400	0
0.6 satd. (Prep. G)	2560	75.0	350	1.4
0.7 satd. (Prep. H)	2890	86.0	250	2.5
0.8 satd. (Prep. I)	2387	86.3	180	3.8
0.9 satd. (Prep J)	1790	86.3	120	4.2

Aspergillus is either very weak or naught[11], but from our experiments it was recognized that some strains such as *Asp. Usamii* R-1031, *Asp. Saitoi* R-1149, *Asp. awamori* R-3523 etc. are as strong as *Asp. oryzae and Asp. sojae.*

Beside, according to the result, superior strains were found in the species of *Asp. Saitoi*, *Asp. awamori*, but not in *Asp. Inuii*, *Asp. Nakazawai*, and *Asp. aureus.*

Mold strains exhibiting the highest proteolytic activity, measured by various assays are summarized in Table II.

Differences in ability to attack different substrates are observed in Table II. For example, *Asp. Saitoi* R-0122 was superior in milk casein digestion but inferior to *Asp. Saitoi* R-0638 in soybean-α-protein digestion, *Asp. Saitoi* R-3221 and *Asp. Saitoi* R-0638 have similar milk casein digestion activities at pH 2.5, but differ in their gelatin liquefaction activities.

Variations in the attack on the same protein substrates were also observed. Thus, *Asp. Saitoi* R-1149 and *Asp. awamori* R-3523 possess similar milk casein digestion ability at pH 2.5, but differ in amino-nitrogen formation.

These dissimilarities in activity suggest the difference in character and composition of proteolytic enzyme systems produced by various strains.

(2) Crystallization of Proteolytic Enzyme from *Aspergillus Saitoi*. Previous reports concerning the crystallization of *Asp. oryzae* proteinase by Akabori[12], Crewther[13], and Miura[14] have been published.

The crude enzyme solution of *Aspergillus Saitoi* R-1149 loses its proteolytic activity remarkably by the following treatment of pH 6.0, 40°C, 1 hr. so it is necessary to adjust the pH of the preparation below 4.5 throughout the whole course of purification.

The crystallization procedure was performed by Akabori's method[12], and amylase which was contained in this preparation was

11) R. Nakazawa and M. Shimo, *J. Agr. Chem. Soc. Japan.* **12**, 931 (1936).

12) S. Akabori, B. Hagiwara, T. Ikenaka and T. Sakota, *Symposia. on Enzyme Chem.* **7**, 50 (1953).

13) W.G. Crewther and F.G. Lennox, *Aust. J. Biol. Sci.* **6**, 428 (1953).

14) Y. Miura, *Ann. Rev. Takamine Lab.* **6**, 14 (1955).

TABLE V

EFFECT OF ACETONE FRACTIONATION OF PREP. H.

Preparation	PU/mg Protein-N.	Yield	Dextrinogenic activity	Glucosidic-bond hydrolysed
Prep. H.	2890	100%	250 min.	2.5%
30% (Prep. K)	—	—	1440	0
40% (Prep. L)	3500	24.2	1440	0
50% (Prep. M)	5290	60.6	1440	0
60% (P.ep. N)	4960	71.1	1200	1.0
70% (Prep. O)	3720	72.3	1200	1.3
80% (Prep. P)	3530	75.2	1000	2.0

removed step by step by starch adsorption, ammonium-sulphate fractionation and acetone fractionation.

a) *Starch adsorption.* The use of potato or wheat starch granule on the elimination of amylase is reported by Schwimmer[15]. Table III shows the effect of starch adsorption of the crude enzyme extract in various concentrations of ethanol.

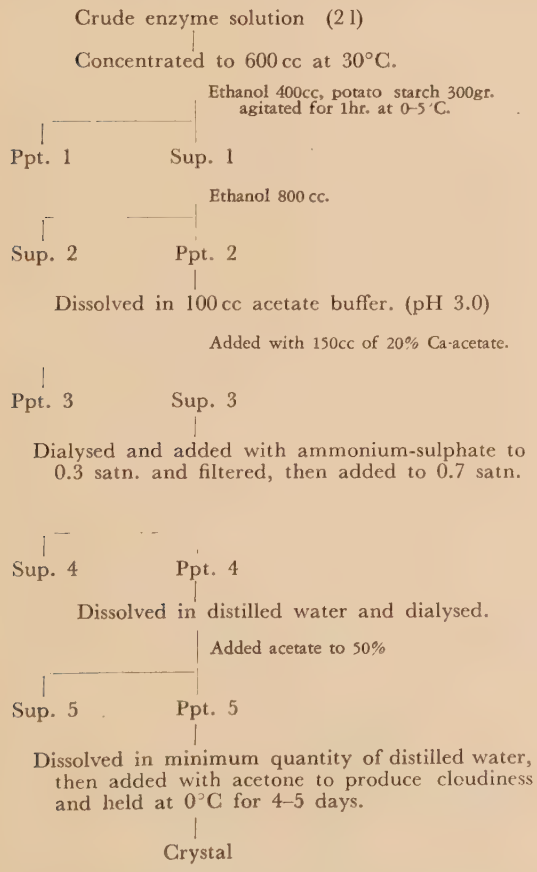


FIG. 1. Crystallization Procedure of *Aspergillus Saitoi* Proteinase.

b) *Ammonium-sulphate fractionation.* Table IV shows the result of ammonium-sulphate fractionation of Prep. D in Table III.

c) *Acetone fractionation.* Table V shows the result of acetone fractionation of Prep. H in Table IV. Prep. M in Table V has scarcely any amylase activity.

d) *Crystallization procedure.* The crystallization procedure is given in Fig. 1. Crystals

FIG. 2. Crystalline Proteolytic Enzyme Obtained from *Asp. Saitoi* R–1149.

are shown in Fig. 2, and the yield and activity of each fraction are summarized in Table VI. Compared with crystalline pepsin, this crystal shows about the half activity in respect of the digestion of milk casein.

15) S. Schwimmer and A.K. Balls. *J. Biol. Chem.* **179**, 1063 (1949).

TABLE VI
YIELD AND ACTIVITY OF EACH FRACTION OF *ASPERGILLUS SAITOI* PROTEINASE

Proteinase	Yield	PU/mg Protein-N.
Enzyme solution	100%	340
EtOH ppt.	83	770
Ca-acetate treated	69	900
Ammonium-sulphate salting out	41	2900
Acetone ppt.	27	5250
Crystal	3	6700

SUMMARY

A procedure for the investigation of mold strains producing acid-stable proteinase is described. One hundred and eighty-nine strains of Black *Aspergillus* were tested. In the over-all pH range, the greatest activity was observed at pH 2.5. Individual mold strains varied in its ability to attack various substrates. Purification and crystallization procedures of the proteolytic enzyme from *Aspergillus Saitoi*, R-1149, are described.

I greatfully thank Prof. K. Sakaguchi and Prof. T. Asai for their guidance and also Dr. M. Mogi for his kind support.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 257~261, 1956]

Studies on the Proteolytic Enzymes of Black *Aspergilli*

Part II. Several Properties of the Crystalline Proteolytic Enzyme Obtained from *Aspergillus Saitoi*

By Fumihiko Yoshida and Michitaro Nagasawa

Noda Industriat and Scientific Research Laboratory

Received July 9, 1956

Many properties of crystalline *Aspergillus Saitoi* proteinase have been studied. This enzyme is not a "metal protein" and its behavior towards several inhibitors resembles that of pepsin.

The properties of the proteolytic enzymes from *Aspergillus oryzae* have been reported by Crewther[1], Wallenfels[2], Kageyama[3], Miura[4], Iguchi[5], Pechmann[6], Matsushima[7], Yasui[8], and others. The authors[9] reported the crystallization of a new proteolytic enzyme from *Aspergillus Saitoi*, and confirmed that this enzyme is different in various respects. In this paper, several properties of this enzyme are described.

METHODS AND MATERIALS

Proteinase Assay. The methods described in the previous report and the modified Anson's method were applied.

Proteinase Preparation. A twice recrystallized preparation was prepared according to the methods described in the previous report was used. The change in activity by the recrystallization was scarcely observed.

Preparation of Substates. Milk casein, hemoglobin and soybean-α-protein used were commercial preparations. Ovalbumin was crystallised from egg-white by the method of Kekwick[10]. In all cases, predenaturation of the substrates was not performed.

RESULTS AND DISCUSSION

1) Shape, Proteinase-unit, Isoelectric Point, and Molecular-weight. The crystals, as shown previous report, are rosettes of needles, the proteinase-unit is 6700 (PU) $\frac{\text{Casein FR.}}{\text{m. eq. tyr./mg. Protein-N.}}$ The isoelectric point is 4.50–4.70 by the method of paper electrophoresis. The molecular weight of the twice recrystallized proteinase calculated was 93,000–98,000 from osmotic pressure by the method of Bull[12].

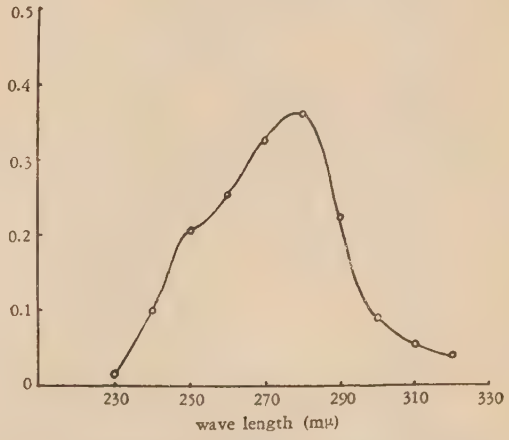


FIG. 1. Adsorption Spectrum of *Asp. Saitoi* Proteinase.

1) W.G. Crewther and F.G. Lennox, *Aust. J. Biol. Sci.* **6**, 410 (1953).
2) K. Wallenfells, *Biochem. Z.* **321**, 189 (1950).
3) K. Kageyama, *Fermentation Technol.* **33**, 54 (1955).
4) Y. Miura, *Ann. Lev. Takamine Lab.* **6**, 14 (1955).
5) N. Iguchi and K. Yamamoto, *J. Agr. Chem. Soc. Japan.* **29**, 88 (1955).
6) E.V. Pechmann, *Biochem. Z.* **321**, 549 (1951).
7) K. Matsushima, *J. Agr. Chem. Soc. Japan.* **29**, 781 (1955).
8) T. Yasui, Lecture delivered at the Agr. Chem. Soc. meeting on Jan. 1955.
9) F. Yoshida, This Bulletin. **20**, 252 (1956).
10) R.A. Kekwick and R.K. Cannan, *Biochem. J.* **30**, 227 (1936).
11) F. Yoshida, This Bulletin.
12) H.B. Bull and B.T. Currie, *J. Am. Chem. Soc.* **68**, 742 (1949).

The ultraviolet adsorption spectrum of this enzyme is shown in Fig. 1. It exhibits a maximum adsorption at 280 mμ which is due to tyrosin and tryptophane.

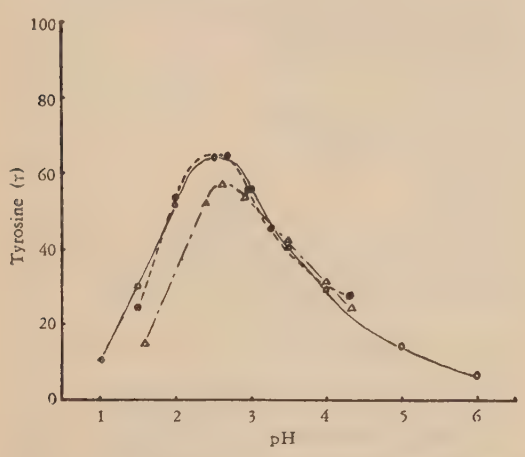


FIG. 2. The Influence of pH and Type of Buffer on the Hydrolysis of Milk Casein by Crystalline *Asp. Saitoi* Proteinase.

—○—○— McIlvaine buffer
··●···●·· citrate-HCl
--△-·-△— glycine-HCl

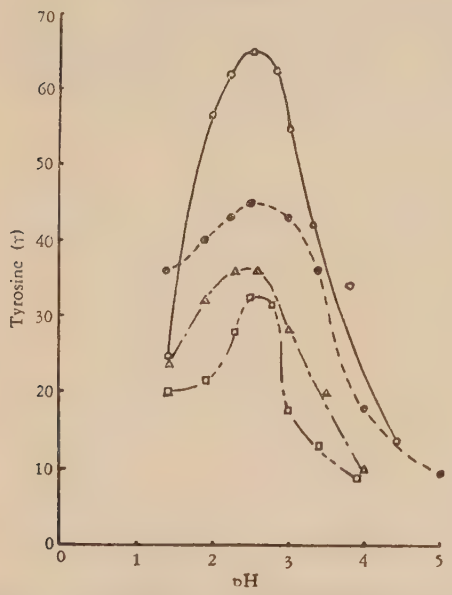


FIG. 3. Hydrolysis of Various Substrates by Crystalline *Asp. Saitoi* Proteinase.

—○—○— milk casein
··●···●·· haemoglobin
--△-·-△— soy-bean-α-protein
··□···□·· ovalbumin

2) Optimal pH. The effect of pH on the rate of hydrolysis was determined for milk casein, soybean-α-protein, hemoglobin and ovalbumin in the range of pH 1.0–6.0 by using the employment of citrate-phosphate, citrate-hydrochloric acid and glycine-hydrochloric acid buffers. In all cases, the optimal pH was found to be in the range of 2.5–3.0 (Fig. 2, Fig. 3).

3) Action on proteins. Fig. 4 and Fig. 5 indicate the increase of amino-nitrogen and trichloroacetic acid soluble nitrogen in the

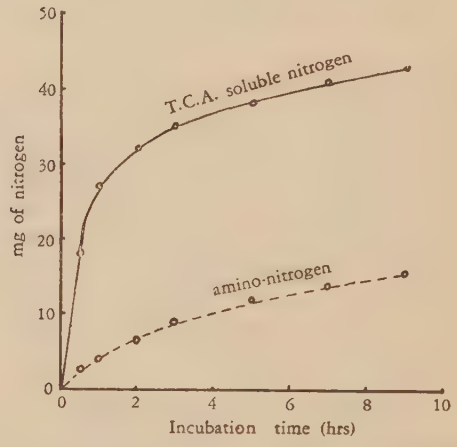


FIG. 4. Hydrolysis of Ovalbumin by Crystalline *Asp. Saitoi* Proteinase.

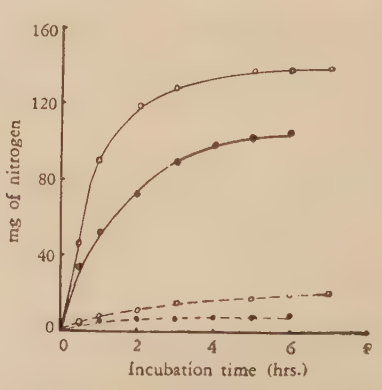


FIG. 5. Hydrolysis of Milk Casein by Crystalline *Asp. Saitoi* Proteinase and Crystalline Pepsin.

····· T.C.A. soluble nitrogen
····· amino nitrogen
○ crystalline *Asp. Saitoi* proteinase (pH 2.5)
● crystalline pepsin (pH 1.5)

TABLE I
RATIO OF TRICHLOROACETIC ACID SOLUBLE-NITROGEN/AMINO-NITROGEN BY VARIOUS HYDROLYSIS

Incubation time	0.5 hrs.	1	2	3	4	5	6	7	8
Ratio of Hydrolysis of									
Milk-casein by *Asp. Saitoi* proteinase	12.3	12.3	10.0	9.1	—	7.2	6.8	6.4	—
Ovalbumin by *Asp. Saitoi* proteinase	8.0	6.3	5.2	3.7	—	3.3	—	3.0	2.5
Milk casein by pepsin	15.6	14.9	15.1	15.1	15.3	15.7	15.3	14.5	—

hydrolysis procedure of ovalbumin and milk casein by crystalline *Aspergillus Saitoi* proteinase and crystalline pepsin.

In the peptic hydrolysis, the ratio of trichloroacetic acid soluble nitrogen/amino-nitrogen is almost constant, while the ratio by *Aspergillus Saitoi* proteinase decreases gradually. (Table I)

From these results, it is implied that *Aspergillus Saitoi* proteinase splits more peptide linkages of protein than pepsin.

4) pH Stability. Fig. 6 shows the effect of pH on the stability of *Aspergillus Saitoi* proteinase in both the presence and absence of the substrate (milk-casein).

5) Heat Stability. The heat stability curve is given in Fig. 7. This enzyme almost loses its activity by the treatment of 55°C, 10 min.

6) Effect of pH and Cations on Thermal Denaturation. The residual activity plotted against time at 55°C, pH 3.0, is shown in Fig. 8. By

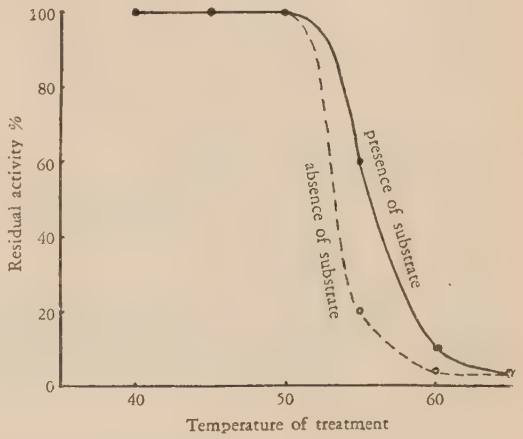


FIG. 7. Loss in Activity of *Asp. Saitoi* Proteinase at Various Temperatures.

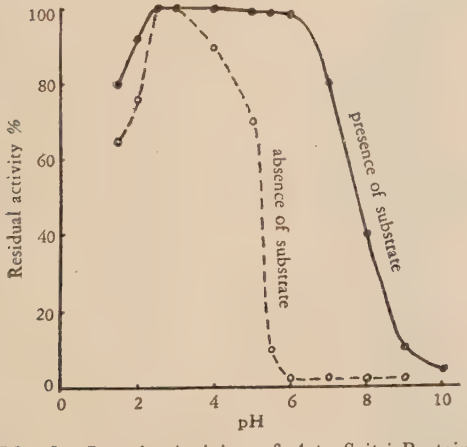


FIG. 6. Loss in Activity of *Asp. Saitoi* Proteinase Solution at Various pH.

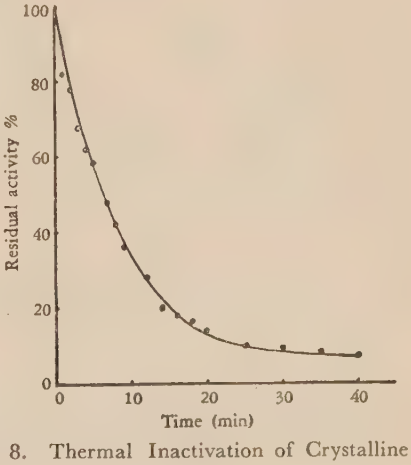


FIG. 8. Thermal Inactivation of Crystalline *Asp. Saitoi* Proteinase at 55°C, pH 3.0.

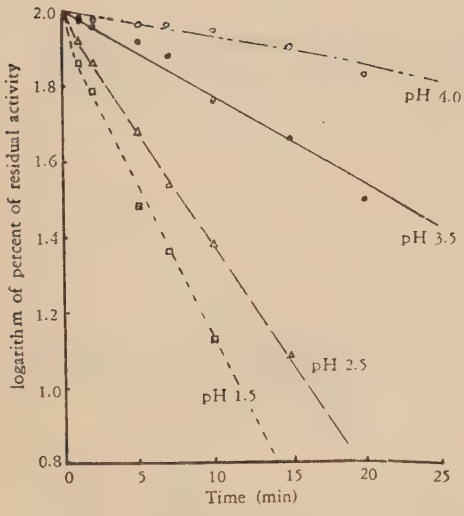


FIG. 9. Thermal Inactivation of *Asp. Saitoi* Proteinase at Various pH.

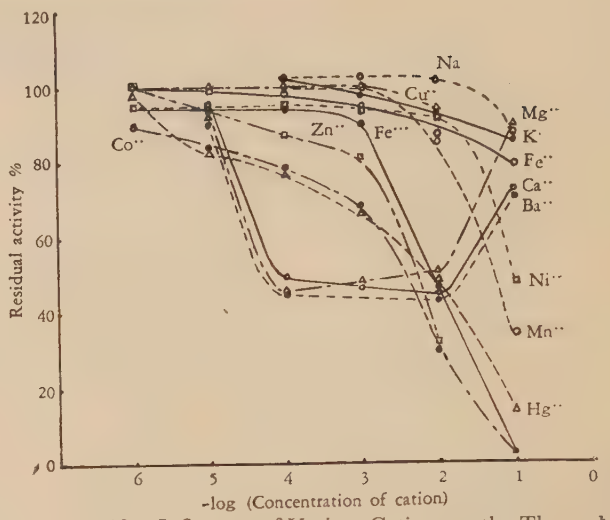


FIG. 10. Influences of Various Cations on the Thermal Inactivation of Crystalline *Asp. Saitoi* Proteinase.

the application of our experimental data to Crewther's equation[12], $(P)=55.7\,e^{-0.0648t}+44.3\,e^{-0.2104t}$ was obtained.

Fig. 9 and 10 show the effect of pH and cations on thermal denaturation and at this condition all cations examined did not appear to have such stabilizing effect at Ca^{++} on trypsin[13,14,15].

7) Inhibitors and Activators. As to the inhibitor of proteinase from *Aspergillus oryzae*, reports by Astrup[16], Crewther[1], and Yasui[8] are published. It seems to be valuable to use the specific inhibitor in order to study the active groups of the enzyme. The effect of various inhibitors, is summarized in Table II.

It has been reported that the acid-proteinase of Taka-diastase is inhibited by EDTA, pyrophosphate, 8-hydroxyquinoline and reactivated by Zn^{++}, Co^{++}, Mn^{++}[17].

Yasui[8] has isolated two types of proteinase from *Aspergillus oryzae*, one of them was inhibited by ascorbic acid, EDTA, $\alpha\alpha'$-dipyridyl

TABLE II

EFFECT OF INHIBITORS AND ACTIVATORS ON THE PROTEINASE OF *ASPERGILLUS SAITOI*

Reagent	Final concentration	Inhibition ratio
I	10^{-2} M	100%
$KMnO_4$	10^{-3}	100
CH_2ICOOH	10^{-2}	5.4
Mercurous acetate	〃	81.3
P.C.M.B.	〃	34.2
$CH_2ClCOOH$	〃	8.2
$CuSO_4$	〃	17.2
$HgCl_2$	〃	35.0
$PbCl_2$	〃	83.6
L-Cystine	〃	9.6
$Pb(CH_3COO)_2$	〃	53.6
L-Cysteine	〃	15.8
Na-thioglycolate	〃	−5.4
Thioglycolic acid	〃	−3.6
Ascorbic acid	〃	0
H_2S	〃	0
8-Hydroxyquinoline	〃	15.4
EDTA	〃	18.8
α-Phenanthroline	〃	21.7
Na-azide	〃	4.9
$\alpha\alpha'$-Dipyridyl	〃	20.9
Na-pyrophosphate	〃	3.8
Na-oxalate	〃	0
Na-fluoride	〃	0
$MnCl_2$	〃	5.4
$ZnCl_2$	〃	10.3
$CoCl_2$	〃	0
$MgCl_2$	〃	0
$CaCl_2$	〃	0
$FeCl_3$	10^{-3}	32.8

13) W.G. Crewther, *Aust. J. Biol. Sci.* **6**, 597 (1953).
14) L. Girini, *Biochim. Biophys. Acta.* **7**, 318 (1951).
15) M. Bier and F.F. Nord, *Arch. Biochem.* **33**, 320 (1951).
16) T. Astrup and N. Alkajaerig, *Nature* **169**, 314 (1952).
17) T. Amano, S. Isojima and H. Fujio, *Med. J. Osaka Univ.* **4**, 255 (1953).

Reagent	Final concentration	Inhibition ratio
Na-laurylsulfonate	10^{-3}	100
Jenus Green	0.01%	7.1
Heparin	〃	37.5
Soybean trypsin inhibitor	〃	0
Pheylhydrazin	10^{-2} M	85.7
Hydroxylamine	〃	73.2
Aniline	〃	96.7
$NaNO_2$	〃	18.2
Phenylenediamine	2×10^{-3}	42.0
Ethylenediamine	10^{-2}	13.0
Sulphanilic acid	〃	23.3
Urea	〃	5.0

while the other was inhibited by iodine.

Our enzyme is neither activated by metal ion nor inhibited by metal-chelating reagent, so the participation of metal is not to be presumed.

Though these results at hand are not sufficient to describe the specificity of the active groups of this enzyme, however, our enzyme compared with the studies on the inhibitors of pepsin by Herriot[18], Shock[19], Schales[20], has a remarkable resemblance to pepsin in its behavior toward various inhibitors which have been used by them. In this respect, the crystalline *Aspergillus Saitoi* proteinase is suggested to have active groups similar to that of pepsin.

18) R.M. Herriot, *J. Gen. Physiol.* **20**, 335 (1936).
19) D. Schock and J.S. Fogelson, *Proc. Soc. Exptl. Biol. Med.* **50**, 304 (1942).
20) O. Schales, *ibid.* **79**, 75 (1952).

SUMMARY

The crystalline proteinase obtained from *Aspergillus Saitoi*, hydrolyses several proteins optimally in the range of pH 2.5–3.0 and splits more peptide linkages than in the case of pepsin.

Thermal denaturation of the enzyme is shown according to the following equation; $(P)=55.7\,e^{-0.0648t}+44.3\,e^{-0.2104t}$ and the thermal inactivation of this enzyme is not stabilized by several cations.

As a result of studying inhibitors this enzyme is not a "metal protein" and in this enzyme the presence of active groups similar to that of pepsin is suggested.

We wish to express our thanks to Prof. K. Sakaguchi, Prof. T. Asai and Assist. Prof. B. Maruo for their kind guidance and also to Dr. M. Mogi for his support.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 262~266, 1956]

Studies on the Proteolytic Enzyme of Black *Aspergilli*

Part III. The Specificity of Crystalline *Aspergillus Saitoi* Proteinase on Synthetic Substrates

By Fumihiko Yoshida and Michitaro Nagasawa

Noda Industrial and Scientific Research Laboratory

Received July 9, 1956

The characterization of crystalline *Aspergillus Saitoi* proteinase by the use of some synthetic peptides and their derivatives was performed. The enzyme possesses a characteristic substrate specificity which different from other known proteinases.

Since it has been shown that well characterized proteinase can hydrolyse synthetic peptides and their derivatives, many reports related to pepsin[1], trypsin[2], chymotrypsin[3,4], papain[5], bromelin[6] etc. have appeared. As for the specificity of proteinases of microorganisms on the synthetic substrates, many interesting reports by Ogle[7], Johnson[8], Amano[9], Iguchi[10], Myceck[11], and others are found.

In order to compare crystalline *Aspergillus Saitoi* proteinase with other known proteinases, the characterization of the enzyme was performed in respect of its specific behavior toward synthetic peptides and their derivatives.

1) J.S. Fruton and M. Bergmann, *J. Biol. Chem.* **127**, 627 (1939).
2) M. Bergmann, J.S. Fruton and H. Pollok, *ibid.* **127**, 643 (1939).
3) M. Bergmann, J.S. Fruton and H. Pollok, *ibid.* **124**, 405 (1938).
4) M. Bergmann, L. Zervas and J.S. Fruton, *ibid.* **111**, 225 (1935).
5) M. Bergmann, J.S. Fruton and H. Pollok, *ibid.* **124**, 321 (1938).
6) M. Bergmann, J.S. Fruton and H. Fraenkel-Conrat, *ibid.* **119**, 35 (1937).
7) J.D. Ogle and A.A. Tytell, *Arch. Biochem. and Biophys.* **42**, 327 (1953).
8) J. Berger, M.J. Johnson and W.H. Peterson, *J. Biol. Chem.* **117**, 429 (1937).
9) T. Amano and S. Isojima, *Med. J. Osaka Univ.* **6**, 81 (1955).
10) N. Iguchi and K. Yamamoto, *J. Agr. Chem. Soc. Japan.* **29**, 394 (1955).
11) M. J. Myceck, S.D. Elliot and J.S. Fruton *J. Biol. Chem.* **197**, 637 (1952).

METHOD AND MATERIALS

Proteinase Preparation. A twice recrystallized enzyme preparation obtained by the method in the previous report[12] was applied here. The proteinous nitrogen content of enzyme preparation was determined by the micro-kjehldahl method.

Preparation of Substrates. All synthetic peptides used were commercial preparations.

Activity Assay. a) Extent of hydrolysis of glycyl-glycine, glycyl-L-leucine, L-leucyl-glycine, glycyl-L-aspartic acid, L-alanyl-glycyl-glycine, L-leucyl-glycyl-glycine was determined by estimating the increase of the liberated $-NH_3^+$ group. For this purpose, the modified alcohol tiration method of Grassmann-Heyde[13] was employed.

b) The extent of hydrolysis of α-benzoyl-L-argininеamide, L-leucineamide, benzoyl-glycineamide was determined by Conway's micro-diffusion technique.

The diffused ammonia was absorbed into N/150 HCl and titrated with N/75 $Ba(OH)_2$.

c) The extent of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine, chloroacetyl-L-tyrosine, carbobenzoxy-L-tyrosine, carbobenzoxy-L-penylalanine was determined by the ninhydrin colorimetry of Troll and Cannan[15].

RESULTS

a) Synthetic Substrates Specificity. The ac-

12) F. Yoshida, This Bulletin. **20**, 252 (1956).
13) W. Grassman and W. Heyde, *Z. Physiol. Chem.* **183**, 32 (1929).
14) F.J. Conway, "Micro-Diffusion Analysis and Volumetric Error" Crosby, Lockwood and Son Ltd. London (1950).
15) W. Troll and R.K. Cannan, *J. Boli. Chem.* **200**, 803 (1953)

TABLE I

ACTION OF *ASPERGILLUS SAITOI* PROTEINASE ON SYNTHETIC SUBSTRATES

Substrates	Substrate concentration	Enzyme concentration Protein-N/ml	pH	Incubation time	Hydrolysis	Remarks
Glycyl-glycine	4×10^{-2} M	222 γ	2.5 4.5	24 hrs.	0% 0	
Glycyl-L-aspartic acid	〃	〃	2.5 4.5	〃	10.4 17.2	
L-Leucyl-glycine	〃	〃	2.5	〃	23.4	Not activated by $Mn.^{++}$
Glycyl-L-leucine	〃	〃	2.5	〃	2.0	
L-Leucyl-glycyl-glycine	〃	〃	2.5 4.5	18	11.5 28.1	
L-Alanyl-glycyl-glycine	〃	〃	2.5 4.5	〃	5.9 7.9	L-Leucyl-↓-glycyl-glycine L-Alanyl-↓-glycyl-glycine
L-Leucineamide	〃	〃	2.5 4.5	24	0 0	
Benzoyl-L-arginineamide	3×10^{-2}	155	2.5 4.5	24	55.3 99.0	
Benzoyl-glycineamide	〃	〃	4.5	〃	0.2	Benzoyl-L-arginine-↓-amide
Carbobenzoxy-L-tyrosine	1×10^{-3}	16	4.5	20	0	
Chloroacetyl-L-tyrosine	〃	〃	4.5	〃	0	
Carbobenzoxy-L-glutamyl-L-tyrosine	〃	〃	2.5 4.5	〃	28.0 58.0	
Carbobenzoxy-L-phenylalanine	〃	〃	4.5	〃	0	Carbobenzoxy-L-glutamyl-↓-L-tyrosine Arrows indicate split positions.

tivities of the enzyme towards several synthetic substrates are summarized in Table I. In all cases, hydrolysis occured in the absence of activators such as HCN, cystein, and ascorbic acid etc.

As it can be observed from the Table I, the proteinase hydrolysed α-benzoyl-L-arginineamide and carbobenzoxy-L-glutamyl-L-tyrosine rapidly and L-leucyl-glycine, L-leucyl-glycyl-glycine, L-alanyl-glycyl-glycine, glycyl-L-aspartic acid slowly whereas this substance did not hydrolyse glycyl-glycine, glycyl-L-leucine, L-leucineamide, α-benzoyl-glycineamide, carbobenzoxy-L-tyrosine, carbobenzoxy-L-phenylalanine. Each of the hydrolysates of L-leucyl-glycyl-glycine, L-alanyl-glycyl-glycine, carbobenzoxy-L-glutamyl-L-tyrosine, L-leucine, L-alanine and L-tyrosine was identified respectively by paper chromatography.

b) Action of Proteinase on α-Benzoyl-L-arginineamide. Fig. 1 and Fig. 2 show the course of hydrolysis of α-benzoyl-L-arginineamide

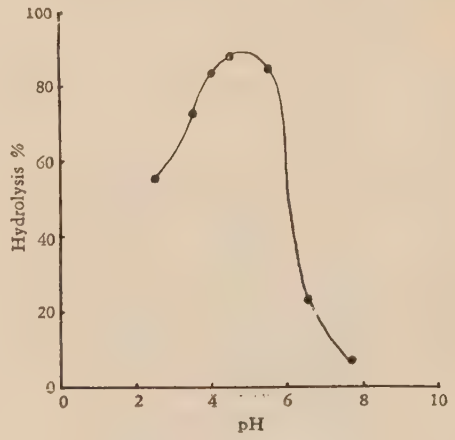


FIG. 1. Effect of pH on the Hydrolysis of α-Benzoyl-L-arginineamide by *Asp. Saitoi* Proteinase.

by this proteinase. The optimal pH for the hydrolysis is in the neighbourhood about of 4.5. At the initial substrate concentration of 0.03 M hydrolysis followed the first order reaction. The velocity constant at 0.03 M was

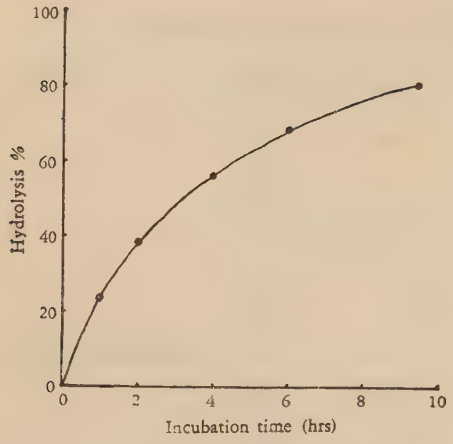


FIG. 2. Hydrolysis of α-Benzoyl-L-Arginineamide by *Asp. Saitoi* Proteinase.

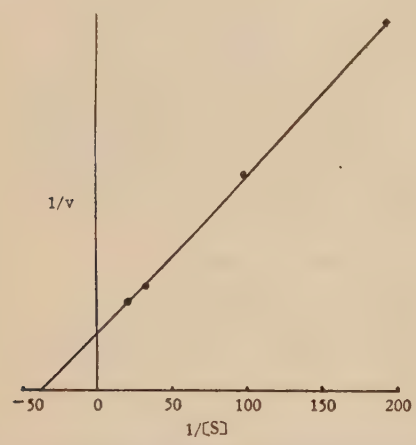


FIG. 3. Effect of Substrate Concentration on the Velocity of Hydrolysis of α-Benzoyl-L-arginineamide by *Asp. Saitoi* Proteinase.

Michaelis' constant is calculated from the following equation.

$1/v = (Km/V[S]) + 1/V$

v; actual velocity
V; maximum velocity
[S]; substrate concentration
Km; Michaelis' constant.

6.1×10^{-5} moles/sec. and the first-order proteolytic coefficient was 1.6×10^{-3} moles/l/min/mg protein-N/ml. Michaelis' constant was $2.5\times10^{-}$ M. when calculated from Fig. 3 and the activation energy for this hydrolysis was 8.83×10^{3} cal/mol, as observed from Fig. 4.

c) Action of Proteinase on Carbobenzoxy-L-glutamyl-L-tyrosine. The optimal pH for the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine was about 4.5 as shown in Fig. 5 and at the initial substrate concentration of 0.001 M the hydrolysis assumed the first-order as shown in Fig. 6. The velocity constant

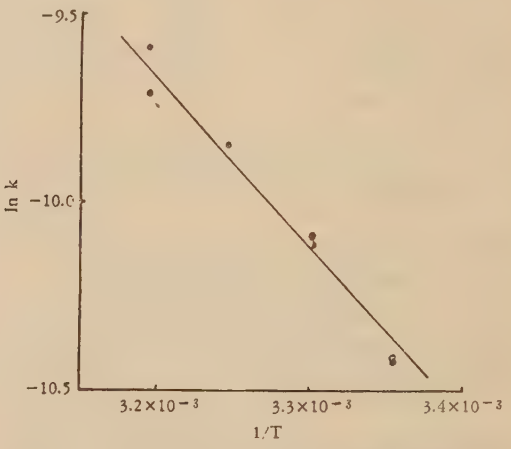


FIG. 4. Determination of Activation Energy of the Hydrolysis of α-Benzoyl-L-arginineamide by *Asp. Saitoi* Proteinase.

Activation energy is calculated from the following equation.

$\ln k = (-Ea/RT) + \text{Constant}$

k; velocity constant
Ea; activation energy
R; gas constant
T; absolute temperature.

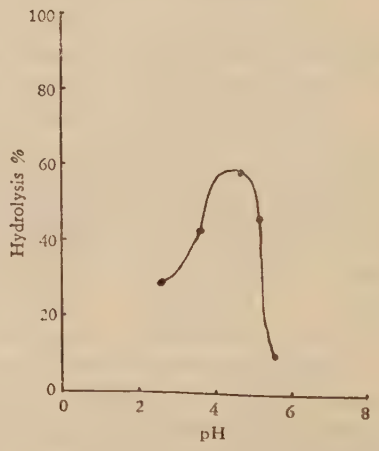


FIG. 5. Effect of pH on the Hydrolysis of Carbobenzoxy-L-glutamyl-L-tyrosine by *Asp. Saitoi* Proteinase.

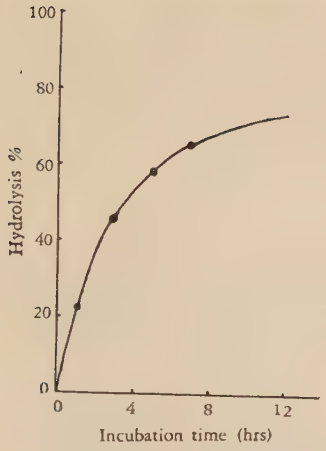


FIG. 6. Hydrolysis of Carbobenzoxy-L-glutamyl-L-tyrosine by *Asp. Saitoi* Proteinase.

was 4.2×10^{-5} moles/sec., and the first-order proteolytic coefficient was 5.3×10^{-3} moles/l/min./mg. protein-N./ml.

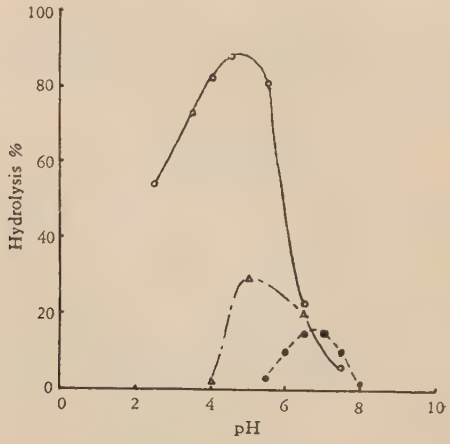


FIG. 7. Hydrolysis of α-Benzoyl-L-arginineamide by Various Mold Proteinases.

Each preparation is adjusted to show the same activity toward milk casein digestion at its optimal pH.

—○—○— *Asp. Saitoi* proteinase
···●···●·· *Asp. oryzae* proteinase
—△—△— *Asp. sojae* wheat bran extract

TABLE II

COMPARISON OF HYDROLYSIS OF VARIOUS SYNTHETIC SUBSTRATES, BY MOLD PROTEINASE

Enzyme	Substrate	Substrate concentration	Enzyme concentration protein-N/ml	pH	Incubation time	Hydrolysis
Asp. Saitoi proteinase	α-Benzoyl-L-arginineamide	3×10^{-2} M	222 γ	4.5	18 hrs.	87.6%
	〃	〃	〃	6.5	〃	23.0
	Carbobenzoxy-L-glutamyl-L-tyrosine	1×10^{-3} M	16	4.5	24	58.0
	〃	〃	〃	6.5	〃	0
Asp. oryzae proteinase	α-Benzoyl-L-arginineamide	3×10^{-2} M	10 mg/ml	4.5	18	0
	〃	〃	〃	6.5	〃	13.7
	Carbobenzoxy-L-glutamyl-L-tyrosine	1×10^{-3} M	1	4.5	24	2.3
	〃	〃	〃	6.5	〃	3.2
Asp. sojae proteinase	α-Benzoyl-L-arginineamide	2×10^{-2} M	10% wheat bran Koji extract	4.5	18	30.6
	〃	〃	〃	6.5	〃	23.0
	Carbobenzoxy-L-glutamyl-L-tyrosine	1×10^{-3} M	1% wheat bran Koji extract	4.5	24	59.4
	〃	〃	〃	6.5	〃	7.5

d) Relations among the Proteinases of *Aspergillus oryzae*, *Aspergillus sojae* and *Aspergillus Saitoi*. Fig. 7 and Table II show the hydrolysis of α-benzoyl-L-arginineamide and carbobenzoxy-L-glutamyl-L-tyrosine by the purified preparation of *Aspergillus oryzae*[16] (including neutral and alkaline proteinases), wheat bran extract of *Aspergillus sojae*, and

16) T. Yasui, Lecture delivered at the meeting of *Agr. Chem. Soc. Japan* 1954.

crystalline *Aspergillus Saitoi* proteinase.

In this case, each preparation was made in order to exhibit almost equal activity toward milk casein at their suitable pH.

DISCUSSION

Crystalline *Aspergillus Saitoi* proteinase hydrolysed α-benzoyl-L-arginineamide and carbobenzoxy-L-glutamyl-L-tyrosine in the absence of activators, α-benzoyl-L-arginineamide was shown as the substrate of trypsin[1] and was hydrolysed by papain[5], bromelin[6], proteinase of *Clostridium histolyticum*[7], Streptococcal proteinase[11], cathepsin II[17] and also carbobenzoxy-L-glutamy-L-tyrosine was hydrolysed by pepsin[1] and cathepsin I[1]. Neutral and alkaline proteinase of *Aspergillus oryzae* differ from our enzyme in their facility of hydrolysis and pH optimum.

In this respect, *Aspergillus Saitoi* proteinase is unique among the crystalline proteinases whose specficity has been examined with known synthetic substrates. However, the crude preparation from *Aspergillus sojae* had similar specificity to the *Aspergillus Saitoi* proteinase, so, the presence of proteinase analogous to *Aspergillus Saitoi* has been presumed in the extract.

17) J.B. Sumner and K. Myrbäck. "*The Enzymes Chemistry and Mechanism of Action*" Vol. 1, 858. (1950).

SUMMARY

The crystalline proteinase from *Aspergillus Saitoi* hydrolysed both α-benzoyl-L-arginineamide and carbobenzoxy-L-glutamyl-L-tyrosine optimally at pH 4.5, and the action on these substrates was studied.

It was recognized that this enzyme is unique among the well-known proteinases. In the wheat bran extract of *Aspergillus sojae*, the presence of proteinase analogous to *Aspergillus Saitoi* is presumed.

We wish to express our thanks to Prof. K. Sakaguchi, Prof. T. Asai and Assist. Prof. B. Maruo for their kind guidance and also to Dr. M. Mogi for his support.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 267~272, 1956]

Studies on Compounds Related to β,-γ-Hexenol, "Leaf Alcohol."

By Yataro Obata and Hiroyuki Horitsu

Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo, Japan.

Received July 12, 1956

In a previous paper[1], the synthesis of β,-γ-hexenol from the Grignard reagent of pentenylbromide and formaldehyde has been reported.

However, it was not established with certainty that the synthetic compound thus obtained was truly β,-γ-hexenol itself. Recently, Harper and Smith traced the procedure outlined in that paper and concluded that the synthetic products were not β,-γ-hexenol and as they mainly contained 2-ethylbut-3-en-1-ol and in addition unsaturated hydrocarbons.

Here again, the earlier procedure was repeated on a larger scale.

From this, it was recognized that β,-γ-hexenol is not to be gained, but 2-ethylbut-3-en-1-ol and unsaturated hydrocarbons are obtained in accordance with the experiment of Harper and Smith[2].

There are a number of opinions in concern of the stereochemistry of β,-γ-hexenol, "leaf alcohol" as to whether this substance is a *cis*-configuration or a *trans*-configuration. Takei and Sakato[3] synthesized 3-hexenol employing the Bouveault-Blanc reduction of ethylsorbate; from the fact that the melting points of the crystalline derivatives of the 3-hexenol were much lower than those of natural "leaf alcohol", the latter was considered to be a *trans*-configuration because its melting points were higher than those of the former. In view of the fact that olefin obtained by the catalytic reduction of the triple bond compound was generally considered to be a *cis*-configuration, Stoll and Rouve[4] synthesized 3-hexenol by catalytic reduction. The resultant hexenol was identical with natural "leaf alcohol", so it was concluded by them that natural "leaf alcohol" was a *cis*-configuration. However, Takei and Ohno[5] showed that a *trans*-configuration was obtained, when the catalyst and temperature were in proper condition. On the contrary, Stoll and Rouve[6] insisted that *trans*-configuration was caused by the difference of the catalyst and catalytic poison, therefore, natural "leaf alcohol" should be a *cis*-configuration. Crombie and Harper[7] obtained 3-hexenoic acid by the Knoevenagel condensation with *n*-butyraldehyde and malonic acid in triethanolamine and the 3-hexenoic acid was reduced to 3-hexenol with lithium aluminum hydride. They concluded that 3-hexenol obtained was different from "leaf alcohol" which was a *cis*-configuration, for the Knoevenagel condensation gave the *trans* double bond according to Lane[8] et al. Moreover, Sondheimer[9] obtained 3-hexenol by the reduction of 3-hexynol with sodium in liquid ammonia. Under such a condition, reduction has been known to give a *trans*-configuration which differed from "leaf alcohol." He showed that the infrared absorption spectrum of the 3-hexenol had a strong band at 10.3μ which is the characteristic band

1) Y. Obata and T. Morito, *J. Agr. Chem. Soc. Japan.*, **25**, 219, (1951).
2) S.H. Harper and J.D. Smith, *J. Chem. Soc.* 1512, (1955).
3) S. Takei and Y. Sakato, *Bull. Inst. Phys. Chem. Research.* **12**, 13, (1933).
4) M. Stoll and A. Rouve, *Helv. Chim. Acta.*, **21**, 1542, (1938).
5) S. Takei and M. Ohno, *Ber.* **73B**, 950, (1940).
6) M. Stoll and A. Rouve, *Ber.* **73**, 1358 (1940).
7) L. Crombie and S.H. Harper, *J. Chem. Soc.* 873, (1950).
8) J.F. Lane et al., *J. Am. Chem. Soc.* **66**, 545, (1944).
9) F. Sondheimer, *J. Chem. Soc.* 879. (1950).

attributed to *trans*-configuration and further showed that hex-3-en-1-ol was obtained by the catalytic reduction of hex-3-yn-1-ol with palladium-calciumcarbonate. This hexenol had a strong band at 13.8μ, which was the characteristic band attributed to a *cis*-configuration and it was proved identical with "leaf alcohol" from the non-depression of mixed melting point test of the crystalline derivatives. Thereof, Sondheimer insisted that natural "leaf alcohol" is a *cis*-configuration. Recently, it has been shown by Harper and Smith that catalytic reduction of hex-3-yn-1-ol with palladium-calciumcarbonate gave *cis*-hex-3-en-1-ol and besides a large quantity of *trans*-isomers. It was found that palladium-bariumsulfate was more selective than palladium-calciumcarbonate. By using this, *cis*-hex-3-en-1-ol was obtained. As a result of the examinning of the infrared absorption spectrum, it was found to possess only one strong band at 13.9μ, and not at 10.3μ. It was compared with the crystalline derivatives of natural "leaf alcohol." Since no depression of mixed melting point test of these derivatives occurred, it was ascertained that natural "leaf alcohol" is a *cis*-configuration. These experiments were repeated by Yukawa et al.,[10] who insisted that the natural "leaf alcohol" was a *cis*-configuration. In view of the fact on the application of the Grignard reaction, mild reaction occurred giving no *trans*-formation for the stereochemical structure, one of the present authors synthesized pent-1-en-3-ol with the Grignard reagent of ethylbromide and acrolein. Upon the interaction of pent-1-en-3-ol with phosphorous tribromide, *trans*-pent-2-enylbromide was obtained through allylic rearrangement; Grignard reagent of this bromide reacted with formaldehyde. As a result, hexenol was obtained. The melting point of the 3,5-dinitrobenzoate of this hexenol thus obtained was identical with that of "leaf alcohol." Therefore, "leaf alcohol" was concluded by one of the present authors to be a *trans*-configuration, because allylic rearrangement gave *trans*-configuration. But the crystalline derivative was refined, and its melting point became higher than that of natural "leaf alcohol." So it seemed to be another substance. However, Harper and Smith repeated the procedure of the present authors and found that the Grignard reagent from *trans*-pentenylbromide reacted with formaldehyde to give 2-ethylbut-3-en-1-ol attributed to the secondary form, no hex-3-en-1-ol attributed to the primary form and in addition unsaturated hydrocarbon as a by-product attributed to the Wurtz-Grignard coupling of *trans*-pent-2-enylbromide. There is a similar case of this abnormal reaction reported by Young and Roberts[11] who have shown that the Grignard reagent from *trans*-but-2-enylbromide reacts with formaldehyde to give only 2-methylbut-3-en-1-ol attributed to the secondary form. Also, with regard to *trans*-3-enylocta-1,5-diene attributed to the Wurtz-Grignard coupling of *trans*-pent-2-enylbromide, there have been obtained by Prevost and Richard[12], 3,7-decadiene, 3-ethyl-1,5-octadiene and 3,4-diethyl-1,5-hexadiene through the Grignard reagent of *trans*-pent-2-enylbromide. So, the present writers insist on the fact that the following reaction processes occur from these results and their own date.

(1) Formation of alcohol (A)

$$CH_3-CH_2-CH=CH-CH_2Br \xrightarrow{Mg} CH_3CH_2-CH=CH-CH_2MgBr$$

(primary form)

$$\rightarrow CH_3-CH_2-CHMgBr-CH=CH_2 \xrightarrow{HCHO} CH_3-CH_2-\underset{\displaystyle CH_2OH}{\underset{|}{CH}}-CH=CH_2 \quad (II)$$

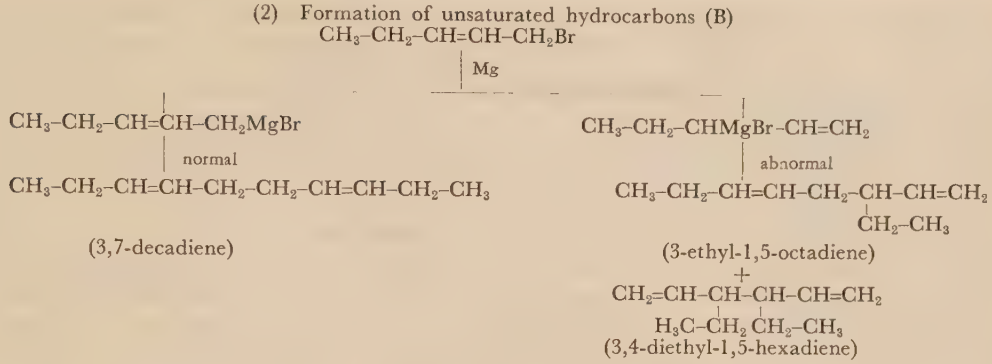
(secondary form)

10) Y. Yukawa et al., *J. Chem. Soc. Japan*, **76**, 5, (1955).

11) W.G. Young and J.D. Roberts, *J. Am. Chem. Soc.* **67**, 148, (1945).

12) C. Prevost and G. Richard, *Bull. Soc. Chim.* **49**, 1368, (1934).

(2) Formation of unsaturated hydrocarbons (B)

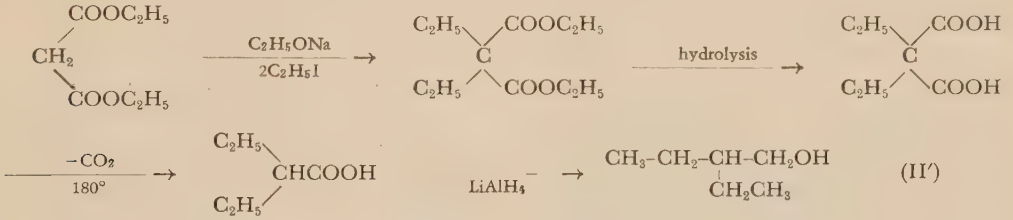


In order to clarify the situation, the writers repeated their experiments. The hexenol obtained from the reaction had boiling point in the range of 137–142°, and refractive index n_D^{20} was 1.4325. It did not possess the characteristic "leaf alcohol" odor. The infrared absorption spectrum is shown in Fig. 1. It shows strong bands at 905 cm^{-1} and 985 cm^{-1} due to vinyl radical $-CH=CH_2$, weak at 690 cm^{-1}, due to *cis*-configuration and at 970 cm^{-1} due to *trans*-configuration. The melting point of the 3,5-dinitrobenzoate was found to be 53°, and that of the 4-diphenylurethane was 79°. These melting points are not identical with those of natural "leaf alcohol" which are 49° and 91°, respectively.

The lack of identity of these alcohols was further demonstrated on admixture, depression of the melting points occurring at 37° and 68°, respectively. Subsequently, this hexenol was catalytically reduced with platinum-black, so it absorbed 1 mole of hydrogen; the product had a boiling point of 149°, and possessed the "camphor" odor.

This saturated hexanol was also found to be not identical with *n*-hexanol in the respect at the boiling point and melting points of crystalline derivatives of 3,5-dinitrobenzoate and 4-diphenylurethane which are 51° and 95°, respectively. These results are identical with published data on 2-ethylbutanol[13] (II′).

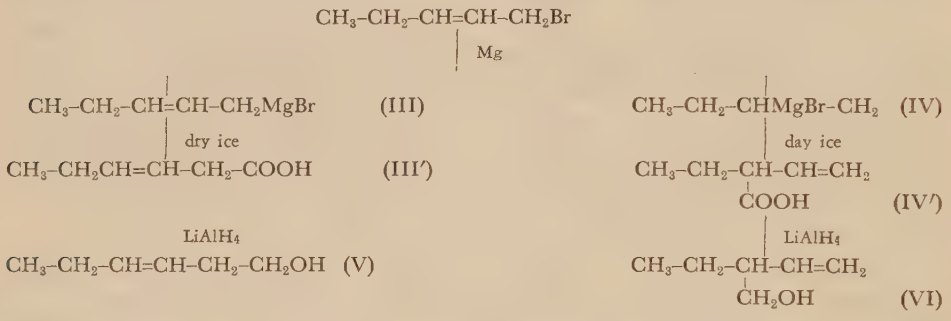
However, according to Harper and Smith's report, the melting point of the 3,5-dinitrobenzoate of the hexanol mentioned above is 35°; this value is not identical with authentic 2-ethylbutanol. Then, the sample of authentic 2-ethylbutanol was synthesized in the following order.



The alcohol obtained possessed the "camphor" odor; it had a boiling point 149°; the refractive index n_D^{20} was 1.4225; the melting point of the 3,5-dinitrobenzoate was found to be 51° while that of the 4-diphenylurethane was 95°. These results are identical with the corresponding values of authentic 2-ethylbutanol. Subsequently, on admixture with these derivatives, no depression occurred, so it was ascertained that the hexenol obtained from reaction (A) is 2-ethylbut-3-en-1-ol. Morever, unsaturated hydrocarbons occurring

13) F.C. Whitmore and F.A. Karnatz, *J. Am. Chem. Soc.*, **60**, 2534, (1938).

with 2-ethylbut-3-en-1-ol had no constant boiling point, in the range of 150–162.5°. These may be a mixture of unsaturated hydrocarbons attributable to the above mentioned reaction (B). Subsequently, in order to ascertain whether the Grignard reagent occurring with reaction (A) is followed by the primary reaction (III) or the secondary reaction (IV), the following procedure was followed. That is, dry ice was employed instead of formaldehyde. Whereupon, hexenoic acid was obtained, and then reduced with lithium aluminum hydride, and the objective 3-hexenol was tried. It has been synthesized previously by Yukawa et al., so their experiment was repeated.



Althogh, the above reaction was considered to have actually occurred, hexenoic acid obtained had boiling point within the range of 97–98°/28 mm Hg, refractive index n_D^{20} was 1.4291, and the melting point of the crystalline *p*-bromophenacylester was 61.5°. These results were neither identical with those of *cis*- nor *trans*--hexenoic acid, because their melting points are 55° and 82.5°, respectively, but these values seemed to be accessible to the *cis*-configuration. The infrared absorption spectrum of the hexenoic acid showed a weak band at 690 cm^{-1}. Hexenol reduced with lithium aluminum hydride had boiling point withih the range of 48–49°/17 mm Hg, refractive index n_D^{20} was 1.4335. The odor did not resemble either natural "leaf alcohol" or 2-ethylbut-3-en-1-ol (II).

The melting point of 4-diphenylurethane was 88.5°, which value seems to be accessible to that of natural "leaf alcohol" being 91°, but on admixture, the melting point was depressed to 67.5°.

The infrared absorption spectrum of the hexenol was not identical with that of natural "leaf alcohol."

Consequently, although the hexenol was reduced catalytically with platinum-black, the odor of *n*-hexyl alcohol did not ensue; its odor was that of "camphor", the same as in the case of 2-ethylbutanol, but as the quantity of the product was scant, distillation or crystalline derivatives were not obtained. According to the above results, the material seemed to be hexenol from the fact that its primary form was scantily produced. Morever, *trans*-hexenol obtained by the Knoevenagel condensation with *n*-butyraldehyde and malonic acid was synthesized using triethanolamine as the reagent for condensation. Upon being reduced with lithium aluminum hydride by Crombie and Harper's method, it yielded *trans*-hex-3-en-1-ol. The odor and the infrared absorption spectrum of it were not identical with those of natural "leaf alcohol"; on admixture, depression of melting point occurred, so it was ascertained that natural "leaf alcohol" is not a *trans*-configuration, but a *cis*-configuration.

EXPERIMENTAL

trans-Pent-2-enylbromide. Pent-1-en-3-ol was prepared by the procedure of Bouis[14]. It had b.p. 112–

14) M. Bouis, *Ann. Chem.*, **9**, 402, (1928).

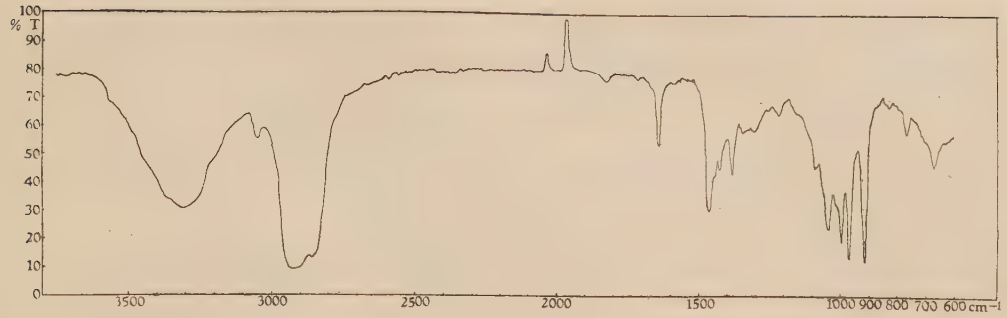


FIG. 1. Infrared Spectrum of Hexenol (II).

115°/757 mm Hg., n_D^{20} 1.4237 (50% yield).

Pent-1-en-3-ol was converted into *trans*-pent-2-enylbromide by the procedure described in the previous report using phosphorous tribromide and pyridine; it had b.p. 26–32°/18 mm Hg., n_D^{20} 1.4732, (30% yield).

Hexenol. Pentenylbromide (57.5 g) was dissolved in dry ether (100 ml) and added for 1 hr. to a stirred suspension of magnesium turning in ether (150 ml). Paraformaldehyde (15 g) was sublimed for 1 hr. into the stirred Grignard reagent. On the next day, the mixture was poured on ice water, decomposed with 30% sulfuric acid, extracted with ether, washed with sodium bicarbonate and dried (Na_2SO_4). After evaporation of the ether, the product was distilled under reduced pressure, to give 40–57°/20 mm Hg., (40% yield). Consequently, distillation gave three fractions at 757 mm Hg., as follows; (i) b.p. 137–140° n_D^{20} 1.4300 (4 g), (ii) b.p. 141–150°, n_D^{20} 1.4335 (4.5 g) and (iii) b.p. 151–162.5°, n_D^{20} 1.4390 (6.1 g).

Of each of the above three fractions, attempts were made to obtain the crystalline derivatives of 3,5-dinitrobenzoate and 4-diphenylurethane. As results, the crystalline derivatives were obtained in the following amount; (i) in large quantity, (ii) scantily, (iii) not at all. Also, upon the measurement of the infrared absorption spectrum showed; (i) absorption corresponding to the hydroxy group, but (ii) scantily, and (iii) not at all. So, it was proved that (i) consisted of an alcoholic product, (ii) was a mixture, and (iii) was unsaturated hydrocarbons. Consequently, the odor of (i) did not resemble of that of natural "leaf alcohol", but had a stimulative odor. The 3,5-dinitrobenzoate was recrystallized from light petroleum and melted at 53°, while, also 4-diphenylurethane melted at 79.5°, and these results were identical with those described in Harper and Smith's report.

Morever, it was observed that the infrared absorption spectrum of (i) as shown in Fig. 1., showed strong bands at 905 cm^{-1} and 985 cm^{-1} due to the vinyl radical, which were weak at 690 cm^{-1} due to *cis*-configuration and at 970 cm^{-1} due to *trans*-configuration. The 4-diphenylurethane of the natural "leaf alcohol" melted at 91°, and the mixed melting point was depressed to 67.5°.

Hexanol. Catalytic reduction of the hexenol in methanol over platinum-black and distillation of the product gave b.p. 149°. The 3,5-dinitrobenzoate and 4-diphenylurethane crystallized from light petroleum gave melting points 51° and 91°, respectively; these values were identical with those of authentic 2-ethylbutanol.

2-Ethylbutanol. Diethylacetic acid was prepared by the procedure of Conrad and Bischoff[15] using diethyldiethylmalonate via diethylmalonic acid; it had b.p. 187–191°/752 mm Hg., (65% yield).

Diethylacetic acid (2 g) was dissolved in dry ether (40 ml), and added to a stirred solution of lithium aluminum hydride dissolved in dry ether (60 ml); after being dropped and stirred further for 1 hr., the material was decomposed with a small quantity of water for excess of lithium aluminum hydride, and poured on diluted hydrochloric acid solution. The ether extract was washed with dilute sodium carbonate and dried (K_2CO_3). After evaporation of the ether, the product was distilled under a reduced pressure to give 53–55.5°/15 mm Hg., n_D^{20} 1.4225. The odor of this alcohol was "camphor" which was the same as the aforementioned hexanol that had been catalytically reduced from the hexenol. Consequently, the 3,5-dinitrobenzoate and 4-diphenylurethane were both recrystallized from light petroleum and gave 50.5°

15) M. Conrad and C.A. Bischoff, *Ann.* **204**, 121, (1880).

and 95°, respectively. These results were identical with those obtained from authentic 2-ethylbutanol, and on admixture of the aforementioned hexanol, no depression was observed. Thus, it was ascertained that the hexenol obtained by the writers' method is 2-ethylbut-3-en-1-ol.

Hexenol by Dry Ice in Place of Formaldehyde. Hexenoic acid was prepared by the procedure of Yukawa et al.; it had b.p. 97–98°/25 mm Hg. n_D^{20} 1.4291; the *p*-bromophenacyl ester gave m.p. 61.5° and differed from corresponding values of *cis*- and *trans*-hex-3-enoic acid which are 55° and 82.5°, respectively.

Hexenol. Hexenol was also prepared by the procedure of Yukawa et al. using lithium aluminum hydride as the reduction reagent; it had b.p. 48–49°/17 mm Hg.; n_D^{20} 1.4335. The odor of the hexenol differed from that of natural "leaf alcohol." The 4-diphenylurethane recrystallized from light petroleum, gave m.p. 88.5° and on admixture with natural "leaf alcohol", depression of b.p. occurred.

Hexanol. Catalytic reduction of the hexenol was performed in methanol over platinum-black, but the product was small in quantity; the odor of the product did not resemble that of *n*-hexanol.

The quantity being scant, no crystilline derivatives could be obtained, but from its odor, it seemed probably to be 2-ethyl-butanol.

***trans*-Hexenoic acid.** This substance was prepared by the procedure of Boxer and Linsted[16], and gave b.p. 100–106°/16 mm Hg.; the *p*-bromophenacyl ester had m.p. 82°.

***trans*-Hexenol.** This substance was prepared by the procedure of Crombie and Harper, and gave b.p. 57.5–58°/17 mm Hg., n_D^{20} 1.4410.

The odor of this hexenol was grassy, but differed from that of natural "leaf alcohol." The 4-diphenylurethane gave m.p. 107°.

The writers wish to acknowledge their indebteness to Mr. Katsumi Yamamoto, of the Central Research Institute, Japan Monopoly Corporation, for the measurement of the infrared absorption spectra and to Dr. Minoru Nakajima, Kyoto University, for the gift of sample of natural "leaf alcohol."

16) S.E. Boxer and R.P. Linsted, *J. Chem. Soc.*, 740, (1931).

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 273~278, 1956]

Studies on Browning Reactions between Sugars and Amino Compounds

Part I. pH Dependency of Browning Interactions between Various Kinds of Reducing Sugars and Amino Compounds

By Hiromichi KATO

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo.

Received July 14, 1956

It was found that primary aliphatic amine reacts with reducing sugars to produce much more brown coloured products than amino acids at the same pH. At 100°C, strong browning interactions were observed in the following cases: first, in an acidic aqueous solution (pH 2–5), between aldoses and aromatic amines; second, in slightly acidic or almost neutral solution (pH 3–8), between aldoses and aliphatic amines or amino acids; third, in an alkaline solution (pH above 8), between aldoses or ketoses and aliphatic amines or amino acids. Not any oxygen is required for these browning reactions. In addition, it is confirmed that the promoting effect of phosphate is remarkable, especially in the range of pH 5–9.

In 1912, Maillard[1] observed that interaction between reducing sugars and amino acids, produces brown products (melanoidin), and in recent years it was demonstrated that this type of reaction plays the most important role in non-enzymatic browning during storage and processing of foods[2]. For this reason, many investigations have been carried out on the browning mechanism of the simple system using both reducing sugars and amino compounds. But due to the complexity of this reaction, the mechanism still remains unknown in many respects.

Wolfrom[3], investigated the pH dependency of the reaction between D-xylose and glycine or L-alanine obtaining results as: acid inhibition (pH 1–3), weak base catalysis (pH 3–5), and strong base catalysis (pH 6.5–8.5).

In the present paper, the effects of pH, concentration, oxygen and phosphate by the use of various sorts of reducing sugars (D-glucose, D-xylose, lactose and D-fructose) and amino compounds (amino acids, aliphatic and aromatic amines) have been investigated.

1) L.C. Maillard, *Compt. rend.*, **154**, 66 (1912).

2) For reviews see: J.P. Danehy and W.W. Pigman, "Advances in Food Research", Vol. III, Academic Press Inc., New York, N.Y., 1951, pp. 241–290; T. Adachi, *Kagaku-no-Ryoiki*, **9**, No. 6, p. 23 (1955).

3) M.L. Wolfrom et al, *J. Am. Chem. Soc.*, **75**, 3471 (1953).

MATERIALS AND METHODS

Materials. All sugars and amino compounds employed here were either G.R. or highly purified. All aqueous solutions were prepared with water doubly distilled by a glass apparatus. Phosphate preparations of Merck and Co. were employed.

Methods. Initial pH of aqueous solutions was adjusted by the addition of sodium hydroxide or glacial acetic acid, in the range of pH 1–3, by the addition of hydrochloric acid. These solutions were taken in test tubes (diameter, 10 mm; length, 10 cm) fixed with air cooler (diameter, 4 mm; length, 50 cm) and heated in a boiling water bath. After cooling, the resulting browning mixtures were diluted to an appropriate optical density with water in the cases of amino acids, because brown substances were soluble in water, and with methanol in the cases of aliphatic or aromatic amines, because they were insoluble in water and soluble in methanol. These diluted solutions were measured by light absorption at 450 mμ, with the help of a Beckmann model DU spectrophotometer.

RESULTS

The mixted aqueous solutions of various

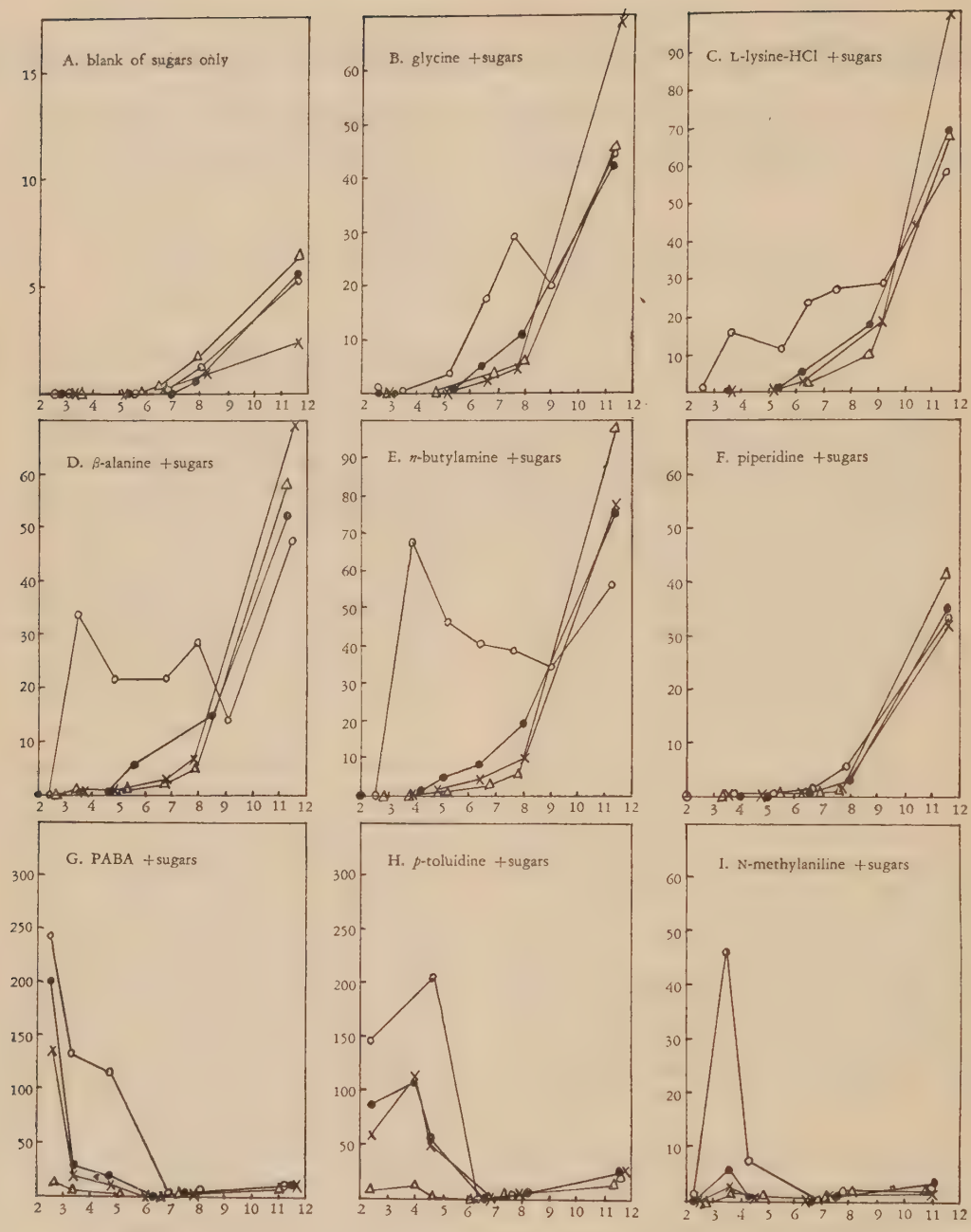


FIG. 1. Relation between pH and the Formation of Brown Substances.

Mixed aq. solns. of sugars (0.25M) and amino compounds (0.5M) heated at 100°C for 60 min. In the range of initial pH 4.5–8.5, M/15 phosphates buffered solution, and in other ranges, only distilled water was used to dissolve materials. spindle: log I_0/I (450 mμ) × dilution; abscissa: initial pH.

—●— glucose, —×— lactose, —○— xylose, —△— fructose.

types of reducing sugars (0.25 M) and amino compounds (0.5 M) were heated at 100°C for sixty minutes at various initial pH. Relation between initial pH and the formation of brown substance is demonstrated in Fig. 1. As for reducing sugars, the following were selected and employed: D-glucose as an aldohexose, D-xylose as an aldopentose, D-fructose as a ketose, lactose as a reducing disaccharide, and as for amino compounds, glycine, β-alanine and L-lysine-HCl as amino acids, *n*-butylamine as a primary aliphatic amine, piperidine as a secondary aliphatic amine, *p*-aminobenzoic acid (PABA) and *p*-toluidine as primary aromatic amines, N-methylaniline as a secondary aromatic amine. Besides, the reactions of ammonia, formamide, urea, triethylamine, diphenylamine and pyridine with reducing sugars were tested. But in the cases of diphenylamine and pyridine no browning reaction occurred, while in the cases of ammonia, formamide, urea and triethylamine only weak reaction occurred.

According to Fig. 1, browning reactions also occur in the cases of primary aliphatic or aromatic amines as well as amino acids.

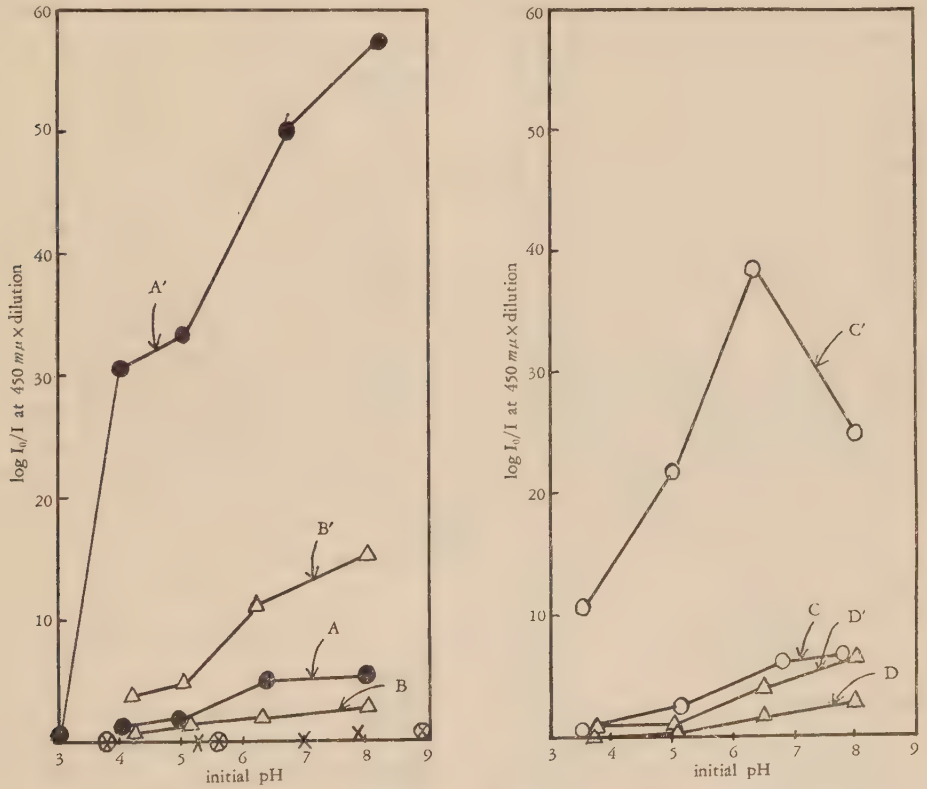


FIG. 2. Relation between Concentration and the Formation of Brown Substances at Various Initial pH.

Mixed aq. solns. of sugars and amino compounds heated at 100°C for 60 min.

A: glucose 0.25 M and *n*-butylamine 0.5 M.
B: fructose 0.25 M and *n*-butylamine 0.5 M.
C: xylose 0.25 M and glycine 0.5 M.
D: fructose 0.25 M and glycine 0.5 M.
×: glucose only, 0.25 M.
A′: glucose 0.5 M and *n*-butylamine 1.0 M.
B′: fructose 0.5 M and *n*-butylamine 1.0 M.
C′: xylose 0.5 M and glycine 1.0 M.
D′: fructose 0.5 M and glycine 1.0 M.
⊗: glucose only, 0.5 M.

The curves of *n*-butylamine are almost similar with those of β-alanine (D & E of Fig. 1). Secondary amines such as piperidine and N-methylaniline have also considerable browning activity. Aromatic amines with aldoses show very remarkable activity in the acid side (pH 2–5), but not remarkable activity in either the neutral or alkali side, and with D-fructose they show only a little activity even in the acid side. Amino acids and aliphatic amines with aldoses as well as with D-fructose have very strong activity in the alkali side (pH above 8), but in either the neutral or acid side (pH 3–8) they have most activity with D-xylose, and considerable activity with D-glucose and lactose, but in case with D-fructose less activity than with lactose.

The difference of activity of aldoses and ketose in neutral and acidic solutions is more clearly shown in Fig. 2. At the concentration of 0.5 M for sugar and 1.0 M for amine, the formation of brown substaces of aldoses (D-glucose and D-xylose) is much larger than that of D-fructose. Namely, in acidic or neutral solution (pH 3–8) aldoses have strong activity with amino acids or aliphatic amines, but ketose has less activity, while on the contrary, in alkaline solution (initial pH 11) aldoses and ketose have almost the same activity. According to this fact, in the cases of amino acids or aliphatic amines, two different reaction mechanisms are present: one is in the cases of acidic or neutral solutions, and the other is in the cases of alkaline solutions.

Summarizing these results described above strong browning interactions occur in the following three cases: first, in acidic aqueous solutions (pH 2–5), between aldoses and aromatic amines as the result of acid catalysis; second, in acidic or neutral (pH 3–8), between aldoses and amino acids or aliphatic amines as the results of inhibition by strong acid and catalysis by weak acid; third, in alkaline (pH above 8), between aldoses or ketoses and amino acids or aliphatic amines as the result of base catalysis.

The influence of oxygen to these three different browning interactions was tested (Table I). The following three methods were applied: (A) heating in a test tube fixed with an air cooler (as used in the experiments represented in Figs. 1 and 2), (B) heating in a sealed tube, and (C) heating in a sealed tube of which the head space was substituted by nitrogen. No significant difference was observed in these three methods. So it may be said that oxygen dose not play any part in these browning reactions.

TABLE I

INFLUENCE OF OXYGEN TO THE FORMATION OF BROWN SUBSTANCES

starting materials	pH min. 0	pH min. 60	Method*	log I_0/I at 450 mμ × dilution
	5.6	5.2	A	1.40
D-xylose+glycine	5.6	5.2	B	1.80
	5.6	5.2	C	1.84
	11.6	9.0	A	43.9
D-glucose+glycine	11.6	9.0	B	43.9
	11.6	9.0	C	42.8
	4.4	4.2	A	8.13
D-xylose+N-methylaniline	4.4	4.2	B	7.89
	4.4	4.2	C	8.25

Mixed aq. solns. of sugars (0.25 M) and amino compounds (0.5 M) heated at 100 C for 60 min.

* A: a test tube fixed with an air cooler.
B: a sealed tube.
C. a sealed tube of which the head space was substituted by nitrogen.

TABLE II

INFLUENCE OF PHOSPHATES TO THE FORMATION OF BROWN SUBSTANCES

	pH min. 0	pH min. 60	log I_0/I (450 m) × dilution	pH min. 0	pH min. 60	log I_0/I (450 m) × dilution
	5.2	5.2	0	5.2	5.4	0
D-glucose only	6.6	5.9	0.17	6.6	6.5	0.04
	7.7	5.9	0.54	7.7	7.4	0.44
	11.5	6.4	5.25	11.5	6.7	10.1
	4.1	4.1	0.86	3.7	3.7	0.15
	5.0	4.8	1.62	5.1	4.8	3.11
D-glucose+*n*-butylamine	6.4	6.0	4.98	6.4	6.0	7.62
	8.0	6.2	5.31	8.0	6.2	17.9
	11.3	8.7	75.2	11.5	8.3	75.4
	4.0	4.0	0.27	4.0	4.1	0.28
lactose+*n*-butylamine	5.1	5.0	0.97	5.3	5.1	1.21
	6.6	6.2	2.79	6.4	6.1	7.95
	8.5	6.4	6.38	8.5	6.4	13.6
	2.5	2.5	0.82	2.4	2.4	0.32
	3.5	3.5	0.47	3.5	3.5	3.71
D-xylose+glycine	5.1	4.6	2.42	5.2	4.8	3.29
	6.8	5.7	6.12	6.6	5.6	17.4
	7.8	5.8	6.62	7.6	6.0	29.2
	9.0	6.1	19.7	9.0	5.8	29.1
	2.4	2.5	0.20	2.4	2.5	0.37
	3.5	3.4	33.6	3.7	3.5	47.2
	4.8	4.6	12.2	4.8	4.3	21.5
D-xylose+β-alanine	6.8	5.3	12.4	6.5	5.2	21.5
	7.4	5.5	13.0	7.9	6.0	28.8
	9.1	5.7	13.4	9.2	5.6	21.5
	11.5	9.4	47.2	11.5	9.2	53.2

Mixed aq. solns. of sugars (0.25 M) and amino compounds (0.5 M) heated at 100°C for 60 min with or without phosphates (M/15).

The influence of phosphate to browning reactions was also tested (Table II). In two cases of sugar-amino compound mixed solutions without phosphate and with M/15 phosphate, very remarkable difference was observed, especially in the range of initial pH 5–9. Therefore, the promoting effect of phosphate is undoubtful.

DISCUSSION

Schroeder and his associates[4] have reported about the browning reactions of glucose and amino acids or peptides at various pH, concluding that amino compound-sugar interaction is limited and only takes place in alkaline solutions. But his experiment was produced in a very diluted concentration, i.e., glucose (0.01 M) and amino compounds (0.05 M). In the present paper, concentration of 0.25 M for sugar and 0.5 M for amino compound were selected. As shown in Fig. 2, by doubling each concentration of sugar and amino compound, i.e., 0.5 M and 1.0 M respectively, the formation of brown substances increases more than four times in the cases of aldoses. Therefore, it is conceived that concentration is a very important factor, and in diluted concentrations the interaction is almost negligible but in high concentrations interaction is undoubtedly present, even in neutral and almost acidic solutions.

In concern of the promoting effect of phosphate, not any definite conclusion have yet been demonstrated. Schwimmer and Olcott[5] did not conclude this on account of difference of the last pH after heating between phosphate buffered and unbuffered solutions.

4) L.J. Schroeder et al, *J. Biol Chem.*, **212**, 973 (1955).

5) S. Schwimmer and H.S. Olcott, *J. Am. Chem. Soc.*, **75**, 4855 (1953).

But from the data of Table II, it is evident that the last pH of buffered solutions after heating, are almost the same as those of unbuffered solutions due to the overload of buffer capacity. Therefore, it can be concluded that phosphates have a strong promoting action for browning reactions.

Acknowledgement. The author wishes to express his hearty thanks to Prof. Y. Sumiki for his guidance throughout this work and is also indebted to Miss. S. Hasegawa for carrying out the spectrophotometric analysis.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 279~283, 1956]

Studies on Browning Reactions between Sugars and Amino Compounds

Part II. Significance of N-glycosides for Browning Reactions

By Hiromichi KATO

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo.

Received July 14, 1956

Aldoses react to form 40–45% N-glycosides with *p*-aminobenzoic acid (PABA) and produce brown substances below pH 6, at 100°C for sixty minutes. But ketoses are inactive under this condition, and N-D-xylosyl-PABA produces the brown substance rapidly than the mixture of D-xylose and PABA. In 0.1 N acidified methanol solution and acetone suspension at room temperature (27°C), N-aldosides of both aromatic and aliphatic amine causes browning to occur faster than the mixture of sugars and amines. Therefore, it was concluded that browning involved in acid-catalytic condition has N-glycosides, as a precursor. While, on the contrary, N-*p*-tolyl-D-isoglucosamine does not make browning at room temperature, even in the acidic condition. N-lactosyl-PABA was synthesized and it was observed that this compound also makes browning under conditions described above.

Many investigators proved that amino-carbonyl condensation reaction occurs in the interaction of sugars with amino compounds[1]. Hodge[2], considered that N-glycosides which have been formed by this condensation reaction undergo the Amadori rearrangement as the next reaction step, products of this rearrangement make browning with amino acids. However, Wolfrom[3] showed that amino compounds promote decomposition of sugars to furfural or other active substances catalitycally, and the carbonyl-amino reaction could occur only to a slight extent if at all in dilute aqueous solutions of aldoses and glycine, although active compounds such as furfural are formed to cause browning with glycine.

In the present paper, the relation between browning and N-glycosides of aliphatic and aromatic amine is described in various acid-catalytic conditions.

Mixed aqueous solutions of PABA (0.25 M) and reducing sugars (0.25 M) (D-glucose, lactose, D-xylose and D-fructose) were adjusted to various initial pH by the addition of sodium hydroxide, and heated at 100°C for sixty minutes (Fig. 1). After heating, log I_0/I at 450 mμ which was adjusted for measurement by dilution with methanol, and the percentage of N-glycosyl-PABA formed, was determined. Inouye[4] and Adachi[1b] showed that the formation of N-glycosyl-sulfanilic acid and N-lactosyl-urea at mild conditions are remarkable in the range of pH 3–4, but their significance for browning is not evident. According to Fig. 1, in the acid side (pH 3–5), much N-glycosides of aldoses are formed (about 40–45%), and browning also occurs remarkably, but, in the case of D-fructose, little N-fructoside is formed with little browning. Therefore, it might be sure to say that N-glycosides have relation to browning reaction in acid-catalytic conditions. As shown by Inouye[4] and in part I of this

1) For reviews see: (a) J.E. Hodge, *J. Agr. Food Chem.*, **1**, 928 (1953). (b) T. Adachi, *Kagaku-no-Ryoiki*, **9**, No. 6, 23 (1955).

2) J.E. Hodge and C.E. Rist, *J. Am. Chem. Soc.*, **75**, 316 (1953).

3) M.L. Wolfrom, et al, *ibid.*, **71**, 3518 (1949).

4) Y. Inouye et al, *J. Agr. Chem. Soc. Japan*, **26**, 360 (1952).

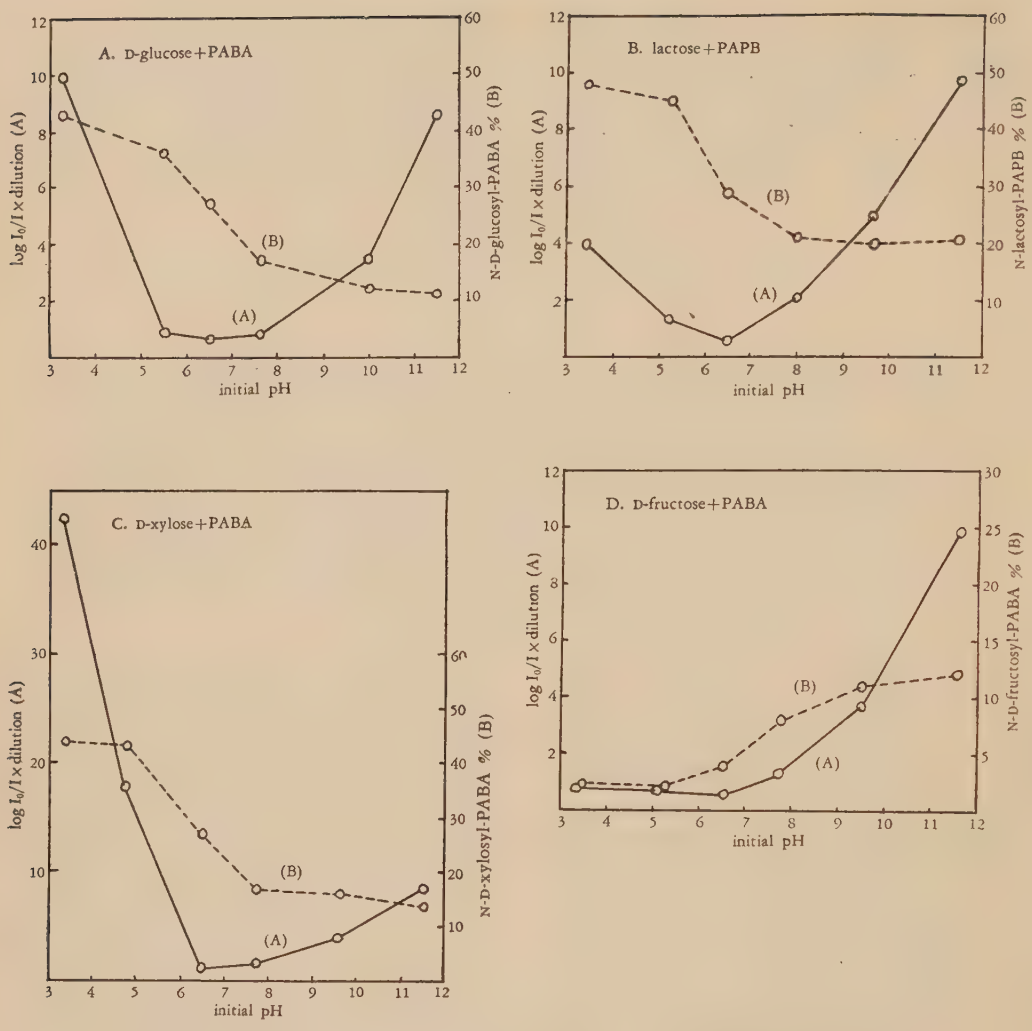


FIG. 1. Relation between Browning and the Formation of N-Glycosides at Various Initial pH.
Mixed aq. solns. of sugars 0.25 M and PABA 0.25 M heated at 100 C for 60 min.
(A); log I_0/I at 450m×dilution.
(B); N-glycosyl-PABA % to free PABA plus N-glycosyl-PABA.

study[5], aldopentoses have strong activity for browning in acidic conditions. It is considered that this phenomenon depends upon the susceptibility of N-pentoside to acids. On the contrary, in the alkali side, there is no relation between condensation reaction and browning (Fig. 1.). Namely, the action of the hydroxide ion to sugars produces browning in this case.

Adachi[6], showed that the formation of the protein-bound-carbohydrate precedes browning reaction in skim-milk heated at 100°, but this result is not satisfactory to decide whether either the free carbohydrate or protein-bound-carbohydrate is a precursor of brown substances. In Table I, comparative

5) The preceding paper of this one.

6) T. Adachi, *J. Agr. Chem. Soc. Japan*, **30**, 378 (1956).

TABLE I

RELATION BETWEEN BROWNING AND THE FORMATION OF N-GLYCOSIDES FOR VARIOUS HEATING TIMES

In the acid side:

starting materials:

	D-xylose+PABA			N-D-xylosyl-PABA		
min.	pH	log $I_0/I \times$ dilution	N-D-xylosyl-PABA %	pH	log $I_0/I \times$ dilution	N-D-xylosyl-PABA %
0	3.3	0	0	3.3	0	100
5	3.3	0.28	47	3.3	0.58	60
10	3.2	1.39	44	3.2	1.56	60
20	3.2	4.48	45	3.2	5.18	57

In the alkali side:

starting materials:

	D-xylose+PABA			N-D-xylosyl-PABA		
min.	pH	log $I_0/I \times$ dilution	N-D-xylosyl-PABA %	pH	log $I_0/I \times$ dilution	N-D-xylosyl-PABA %
0	11.1	0	0	11.1	0	100
5	6.9	2.11	0	9.6	0.46	98
10	6.7	2.42	2	8.9	0.78	91
20	6.6	3.28	8	7.3	1.08	82
40	6.3	3.87	12	6.9	1.68	41

Mixed aq. soln. of D-xylose 0.25 M and PABA 0.25 M or aq. soln. of N-D-xylosyl-PABA 0.25 M heated at 100 C. log I_0/I measured at 450 mμ.

experiments employing N-D-xylosyl-PABA and the mixture of D-xylose and PABA as starting materials were carried out. Each 0.25 M solution or mixture was heated at 100°C for different times. In the alkali side of initial pH 11.1, it is evident that free D-xylose causes browning by the action of the hydroxide ion in accordance with the result of Fig. 1 and N-D-xylosyl-PABA is rather stable and does not cause browning. However, in the acid side of initial pH 3.2, N-D-xylosyl-PABA produces much more brown substance than a mixture of D-xylose and PABA, so N-D-xylosyl-PABA might be a precursor of brown substance. But, for the reason of the rapid hydrolysis of N-glycosyl-PABA in acidic condition, this conclusion is not always evident. Experiments quite the same with Table I were carried out employing N-*n*-butyl-D-glucosylamine as a N-glycoside of aliphatic amine, but resulted in failure on account of little difference of the brown color produced between a mixture of D-glucose and *n*-butylamine and N-*n*-butyl-D-glucosylamine of which more rapid hydrolysis than N-glycosyl-PABA occured, even in the alkali side[7].

More definite data for the conclusion described above in which N-glycosides are a precursor of brown substances for the first reaction stage are presented in Table II. In acidic methanol solutions or acetone suspensions, various N-glycosides, mixtures of sugars and amino compounds and N-*p*-tolyl-D-isoglucosamine, were allowed to stand at room temperature (27°C) to obtain the result that N-glycosides produce far too much brown substances than the mixtures of two conponents. According to Table II, in the cases of N-glycosides of aromatic amines (*p*-toluidine and PABA), the difference is very remarkable, and N-*n*-butyl-D-glucosylamine also produces much more brown substance than the mixture of two components, although difference is smaller. In striking contrast with this, N-*p*-tolyl-D-isoglucosamine

7) W. Pigman et al, *J. Am. Chem. Soc.*, **73**, 1976 (1951).

TABLE II

BROWNING OF VARIOUS N-GLYCOSIDES OR MIXTURE OF SUGARS AND AMINO COMPOUNDS IN ACIDIC ORGANIC SOLVENTS AT ROOM TEMPERATURE

starting materials:	conc. (M)	solvent	acid added (0.1N)	hours	log I_0/I at 450 mμ × dilution
N-*p*-tolyl-D-glucosylamine	0.1	MeOH	conc. HCl	20	7.92
D-glucose+*p*-toluidine	each 0.1	MeOH	conc. HCl	20	0.08
N-*p*-tolyl-D-isoglucosamine	0.1	MeOH	conc. HCl	20	0
N-D-glucosyl-PABA	0.05	MeOH	conc. HCl	20	6.17
D-glucose+PABA	each 0.05	MeOH	conc. HCl	20	1.67
N-D-xylosyl-PABA	0.01	MeOH	conc. HCl	20	0.11
D-xylose+PABA	each 0.01	MeOH	conc. HCl	20	0.01
N-D-xylosyl-PABA	0.1	acetone	conc. HCl	20	59.8
D-xylose+PABA	each 0.1	acetone	conc. HCl	20	5.82
N-D-xylosyl-PABA	0.1	acetone	CH_3COOH	20	0.01
N-D-glucosyl-PABA	0.1	acetone	conc. HCl	20	46.3
D-glucose+PABA	each 0.1	acetone	conc. HCl	20	2.17
N-lactosyl-PABA	0.1	acetone	conc. HCl	48	4.51
lactose+PABA	each 0.1	acetone	conc. HCl	48	0.03
N-lactosyl-PABA	0.1	MeOH	conc. HCl	48	0.25
N-*n*-butyl-D-glucosylamine	0.1	MeOH	CH_3COOH	48	0.40
D-glucose+*n*-butylamine	each 0.1	MeOH	CH_3COOH	48	0.19
N-*n*-butyl-D-glucosylamine	0.1	MeOH	conc. HCl	48	0
D-glucose+*n*-butylamine	each 0.1	MeOH	conc. HCl	48	0
N-*n*-butyl-D-glucosylamine	0.1	acetone	CH_3COOH	48	2.75
D-glucose+*n*-butylamine	each 0.1	acetone	CH_3COOH	48	2.40
N-*n*-butyl-D-glucosylamine	0.1	acetone	conc. HCl	48	1.31
D-glucose+*n*-butylamine	each 0.1	acetone	conc. HCl	48	0.02

Methanol homogeneous solutions or acetone suspensions acidified to 0.1 N with conc. HCl or glacial acetic acid allowed to stand at 27°C.

is very stable and almost no browning was observed in the same condition. So, it is evident that N-*p*-tolyl-D-glucosylamine causes browning via a route which differs from the Amadori rearrangement. However, it was observed that at a higher temperature (100°C) in aqueous solution, this compound produced much more brown substance than N-*p*-tolyl-D-glucosylamine which was in accordance with Gottschalk's report[8]. Therefore, in some cases, the Amadori rearrangement product gives a remarkable browning reaction, but, in general, the rearrangement is not always necessary for browning.

N-Lactosyl-PABA, a new compound, was prepared, and considerable browning occured in 0.1 N acidified acetone suspension by the addition of hydrochloric acid at 27°C, although the reaction velocity was much slower, but, in an acidic methanol solution very little browning occurs. In this case, concerning the browning mechanism, no evidence could be obtained whether browning occurs after or before hydrolysis of glucose-galactose linkage.

Finally, as shown in Table II, in the cases of N-glycosides of aromatic amines, 0.1 N hydrochloric acid is far more effective for browning than 0.1 N acetic acid, but, on the contrary, in the case of N-*n*-butyl-D-glucosylamine, acetic acid is more effective than hydrochloric acid. These facts seem to correspond to the results of part I of this study[5] in which the appropriate pH for browning was 2–5 between aromatic amines and aldoses and it was 3–8 (a milder acidic pH) between aliphatic amines or amino acids and aldoses.

EXPERIMENTAL

A. Preparation of N-Contained Sugar.

N-D-Glucosyl-PABA[9], (dec., 130°C), N-D-xylosyl-

8) A. Gottschalk, *Biochem. J.*, **52**, 455 (1952).

9) Y. Inouye et al, *J. Agr. Chem. Soc. Japan* **25**, 59 (1951–52).

PABA[9] (dec., 174–175°C), N-*p*-tolyl-D-glucosylamine[10] (dec., 111–112 C), N-*p*-tolyl-D-isoglucosamine[11] (dec., 153–154°C), N-*n*-butyl-D-glucosylamine[7] (dec., 89–90°C), were prepared by methods cited in the literature, and identified by m.p.

Preparation of N-Lactosyl-PABA. Lactose, 3 g, was dissolved in 6 cc hot water and to this solution 54 cc of 99% ethanol which had dissolved 1.15 g PABA and 0.15 g ammonium chloride was added. The resulting solution was refluxed in a water bath rapidly before lactose began to crystallize out for twenty minutes. After cooling, the unreacted lactose which crystallized out was filtered and the filtrate was allowed to stand in an ice box. Fine needles were obtained and washed with ethanol and ether; yield, 2.0 g (51%). Recrystallization from 90% ethanol raised m.p. 197–198°C (dec., in a sealed tube). $[\alpha]_D^{27}$ −6.7 →+2.8 (in aq. NaOH, pH 6.2).

Anal. Calcd. for $C_{19}H_{27}NO_{12}\cdot H_2O$: C, 47.60; H, 6.05; N, 2.92. Found: C, 46.86; H, 5.94; N, 2.88%.

B. Measurements of the Formation of N-Glycosyl-PABA.

Inouye's method[12] was adopted. The solution contained PABA or its N-glycoside of which 0.0045 cc was spotted on a paper strip, developed with phenol-0.1% aq. NH_4OH (10%), and then dried. The corresponding parts of PABA and N-glycosyl-PABA were cut in 5 cm-length, extracted twice with 5 cc of 50% aq. methanol, and to the extracts 1 cc of 3% H_2SO_4 acidic 0.5% *p*-dimethylaminobenzaldehyde 50% aq. methanol solution was added to color, and the absorption at 450 mμ was measured by a Beckmann model DU spectrophotometer. The percentages of N-glycosyl-PABA to PABA plus N-glycosyl-PABA on a same strip were obtained. The colored solution in this method was unstable and reduced the absorption by light, but after exposition for a certain time it remained almost unchanged resulting contrary to Inouye's report[12] and the standard curve also became linear. So that, in this experiment, measurements were made with the solutions being allowed to stand in an ordinary place for twenty-four hours after color reaction.

Acknowledgement. The author wishes to express his sincere gratitude to Prof. Y. Sumiki for his guidance throughout this work. He is indebted to Miss. S. Hasegawa for the spectrophotometric analyses and also to Mrs. Sato, Miss. Isobe and Miss. Suzuki for the micro elementary analyses.

10) F. Weygand, *Ber.*, **72**, 1663 (1939).
11) F. Weygand, *ibid.*, **73**, 1259 (1940).
12) Y. Inouye et al, *J. Agr. Chem. Soc. Japan*, **27**, 1 (1953).

[Bull. Agr. Chem. Soc. Japan, Vol. 20, Suppl., p. 284~288, 1956]

Alterations of Metabolism in Plants at Various Temperatures

Part I. Mechanism of Cold Damage of Sweet Potato

By Hitoshi Shichi and Ikuzo Uritani

Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University.

Received July 19, 1956

Some metabolic changes occuring in sweet potatoes stored at 0°C and 20°C were studied. Sweet potato when stored at 0°C, seems to have a critical point at which a peak of the respiration curve can be observed and the effect of DNP* on oxygen uptake also starts to decline, the pH values begin to rise and the amount of Pi shows a down-hill decrease accompanied by concomitant increase in that of Po. The results obtained here were accounted for as possible blocks in the respiratory system due to the deterioration of cytoplasm caused by the low temperature treatments. Cold damage of the cell membrane was qualitatively suspected from the readiness of penetration of *Rhizopus nigricans* into the cell. These noticeable changes of cellular metabolism were not observed in both the control of sweet potato stored at 20°C, and in potato tubers kept at 0°C and 20°C.

Plants are in general susceptible to freezing injury. Some plants are known to be damaged by merely keeping at low temperatures, slightly above the freezing point[1,2]. The temperature at which a plant is affected varies from plant to plant, and also with advance of physiological age. For instance, the rice plant entering the initial period of heading or fruiting is so sensitive to tempertures below 20°C, that it is sometimes subjected to serious damage. Sweet potatoes as well as pumpkins, stored at 0°–7°C are easily infected with soft rotten disease. Although such low temperature damages of various plants can not be accounted for by a simple and identical mechanism, many evidences allow us to consider that they must have a great deal of common characteristics. For the purpose of studying the effect of the cold on plants, to begin with, we have attempted to investigate some biochemical changes in the sweet potatoes which have been stored at low temperatures.

It is notorious that the sweet potatoes damaged by cooling at 0°–7°C, for several months suffer ready infection of soft rot. The weakness of sweet potato to low temperatures seems to be due to the fact that it comes from the tropics. The same can be said of the susceptibility of banana and papaya to the cold.

Iwasaki[3] and Matsumoto[4], found a reduction in catalase activity, a decrease in pH and an accumulation of sugars upon the exposure of sweet potato to low temperatures. Harter et al[5] and Iwasaki[6], suggested that one of the pathogenic factors of *Rhizopus nigricans*, a fungus which causes sweet potato soft rot, must be a pectinase. However, there is not much information available at present to explicate the mechanism of cold injury and how the soft rot disease progresses in the damaged sweet potato.

* The following abbreviations are employed: DNP = 2,4-dinitrophenol, Pi = inorganic phosphate, Po = organic phosphate, ADP = adenosine-diphosphate and ATP = adenosinetriphosphate.

1) P.W. Went; *Ann. Rev. Plant Physiol.*, **4**, 347 (1953).

2) W.T. Pentzer; *Ann. Rev. Plant Physiol.*, **5**, 205 (1954).

3) Y. Iwasaki; Nogaku (Agriculture), **1**, 470 (1947).

4) K. Matsumoto; Nogyo oyobi Engei (Agriculture and Horticulture) 21, 27, 83, 115 (1946).

5) L.L. Harter and J.L. Weimer; *J. Agr. Res.*, **22**, 371 (1921).

6) Y. Iwasaki, Y. Tanaka et al; Report of Agriculture Institute of Japan, No. 1 (1947).

METHOD

Among some accessible varieties of sweet potato, Okinawa No. 100 was chosen because of its high susceptibility to the cold. For comparison, potato which is known to be more resistant to the cold than sweet potato was used. A sweet potato root and a potato tuber were cut in halves respectively. One half of each sample was stored at 0°C and the other at 20°C, for a certain length of time. There was no fear that the one stored at 0°C might freeze and be damaged, as the freezing point of sweet potato was in the lower range from $-1.5°\sim2.0°C$.

Measurement of Respiration. Respiration was measured according to Beevers[7]. A number of small discs were made (7 mm diameter, 0.5-mm thick of which 20 discs were placed in a Warburg flask containing 1.5 ml of 0.03 M phosphate buffer (containing 0.5% sucrose) and 0.5 ml of water, and the oxygen uptake was measured at 25°C for 90 min. The effect of 2,4-dinitrophenol was also tested under the same conditions except that water was replaced by a DNP solution of which the final concentration was made up to 5×10^{-5} M.

Analysis of Phosphate Compounds. Both acid soluble organic and inorganic phosphates in the tissues were fractionated according to Schneider[8], Ogur et al[9] and Akazawa et al[10] with some modifications as follows: Five g. of tissues was homogenized with 25 ml of 12% $HClO_4$ and centrifuged at 3000 r.p.m. for 15 min. The residue was reextracted with 5 ml of 6% $HClO_4$, and centrifuged off. The supernatant fractions obtained, were combined and provided for analysis of the phosphates. An aliquot was taken and Pi was measured according to Nakamura's method. For the determination of Po, an aliquot was digested with 60% $HClO_4$ and all of the organic phosphates were determined in the form of Pi, the difference of the amounts of Pi before and after digestion thus indicating the amount of Po in the test solution.

Measurement of pH. Tissues were minced and the pH was promptly examined with pH testing papers.

Water Absorption Test. By reference to the experiments carried out by Thimann et al[11] on the phytohormone-treated potato discs, the experiments were carried out as described below. A number of discs (1.5 cm diameter, 1-mm thick) were prepared with the aid of a handmicrotome and rinsed in tap water for 20 hrs. Twenty discs which arrived at the equilibrium state were sampled. In order to remove the water that adhered to the surface, the discs were gently squeezed between two sheets of filter paper for ten sec. with a substance weighing 100 g. The total-weight of the 20 discs was recorded prior to carrying them into a schale containing a 10 p.p.m. alphanaphthaleinacetate solution (pH 5.4). The discs were placed in the hormone solution so as to have the upper side exposed to the air and the back-side steeped in the solution by making use of a set of wire gauze. After a 48 hr.-incubation at 25°C, the fluctuations in weight were measured.

Inoculation of *Rhizopus Nigricans*. The spore suspension of *Rhizopus nigricans* was inoculated on the surface of the sliced samples and incubated at 25°C, under water-saturated condition. Four varieties of the fungus were compared with respect to their ability of penetration.

RESULTS

1) Respiratory Changes and the Effect of DNP. When the oxygen uptake of the sweet potato kept at 0°C was compared with that of the sample stored at 20°C, it was found that the former exceeded the latter at the 8th—the 10th day, but declined rapidly thereafter. This period of 8–10 days after cold treatment seems to be the critical point at which some biochemical changes occur in the cold-treated sweet potato. Addition of DNP caused a constant increase of 100–120% in respiration of the sample kept at 20°C, while in the sample treated at 0°C, the degree of respiratory increase began to decrease at an elapse of 8–10 days and finally dropped down to the level of 30–10% (Fig. 1).

Respiration in the organisms couples to the following phosphorylation reaction.

$$ADP+Pi \longrightarrow ATP$$

As the respiratory rate is dependent upon the ratio of ADP/ATP[12, 13, 14, 15], increase in

7) H. Beevers; *Am. J. Bot.*, **40**, 91 (1953).
8) W.C. Schneider; *J. Biol. Chem.*, **161**, 293 (1945).
9) M. Ogur and G. Rosen; *Arch. Biochem.*, **25**, 262 (1950).
10) T. Akazawa, I. Uritani; *J. Biochem.*, in press.
11) D.P. Hackett and H.A. Thimann; *Am. J. Bot.*, **39**, 554 (1952).
12) L. Lynen; *Ann.*, **546**, 120 (1941).
13) M.J. Johnson; *Science* **94**, 200 (1941).
14) P. Siedevitz and V.R. Potter, *J. Biol. Chem.*, **201**, 1 (1955).
15) A. Millerd and J. Bonner, *J. Histo. Cytochem.*, **1**, 254 (1953).

the concentration of ADP in the reaction system causes acceleration of the respiratory rate. ATP given rise to by the coupled reaction of respiration and phosphorylation

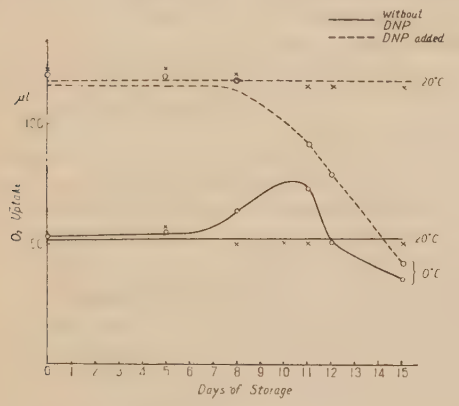


FIG. 1. Respiratory Change Related to the Duration of Storage.
Curves were made on the basis of O_2 uptake for 90 min at 25 C.

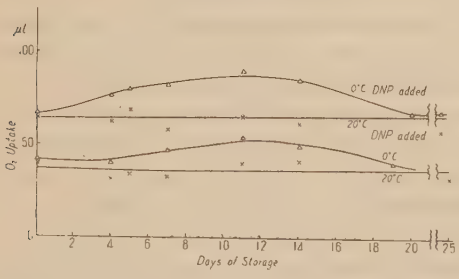


FIG. 2. O_2 Uptake of Potato-disks Measured at 25°C for 90 min.

will be consumed by the ATP-utilizing systems in vivo and the resultant ADP will be returned to the ADP pool to sustain its concentration at a constant level. DNP which is known as an uncoupling agent of the oxidative phosphorylation results in an increase in the ADP/ATP ratio, followed by a respiratory increase.

In view of the effect of DNP on oxygen consumption, the in vivo ratio of ADP/ATP can therefore be suspected.

At 8–10 days elapse, the oxygen uptake shows a temporary rise while the effect of DNP becomes less remarkable, a fact suggesting that the ratio of ADP/ATP will be high in this period. It may be that such an increase in the ADP level is due to the cold damage of the cellular particulates which results in the activation of ATPases which have been inactive when in a bound state to the normal particulates, or leads to respiration independent of the phosphorylation system. The damage of the respiratory system after 8–10 days seems to be responsible for the rapid fall in respiration and the effect of DNP. An enzyme taking part in the respiratory system and most labile to the cold effect may be cytochrome oxidase since, it is suggested that this enzyme is dependent on the normal structure of mitochondria.

In the case of potato, no significant difference of respiration was observed between the samples kept at 0°C and at 20°C, although a slight increase in respiration was observed with the sample kept at 0°C. Addition of DNP caused neither rise nor fall in respiratory increase of all the samples (Fig. 2).

2) Change of Phosphate Components. As shown in Fig. 3, not any noticeable change

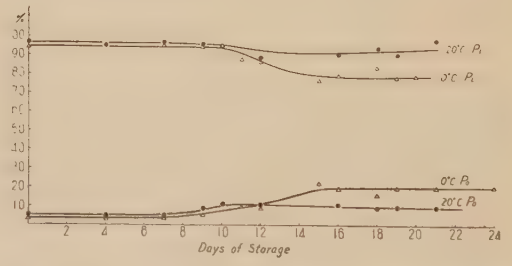


FIG. 3. Change of Acid Soluble Pi and Po related to the Duration of Storage.

$$\text{Expressed as: } \% = \frac{\text{Pi (or Po)}}{\text{Total amt. of acid solu. P}} \times 100$$

was observed with respect to both Pi and Po, during a 8–10 day storage.

Pi however, showed a down-hill decrease accompanied by the concomitant increase in Po. It is considered that most of the acid-soluble organic phosphates consist of glycolysis intermediates such as glucose-1-phos-

phate and glucose-6-phosphate.

As enzymes capable of degrading starch, amylase and phosphorylase are contained in plants, of which the latter having a smaller temperature coefficient (Q_{10}), seems to be in an active state even under cold conditions. Bonner et al[16], for instance, consider phosphorylase to be the enzyme that acts on starch giving rise to monophosphate esters, intermediate metabolites for sucrose formation in potato tuber, kept at low temperatures.

3) Rise in pH Values. The potato tuber kept at 0°C for either a longer or shorter period gives a constant pH value of 5.5, while in sweet potato root stored at 0°C, pH values begin to ascend at the 10th day and

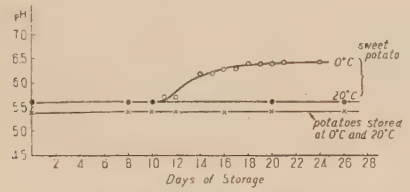


FIG. 4. Change of pH Related to the Duration of Storage.

attain a pH value as high as 6.5 (Fig. 4). It has been proved that sweet potato has the tricarboxylic acid cycle and contains a large amount of organic acids such as citrate and succinate[17], which are well-known intermediates of the TCA cycle. The rise in pH values in cold-treated sweet potato may be due to reduction in the level of these organic acids. In addition, the rise in pH was observed in parallel with the decrease in oxygen uptake.

This phenomenon, therefore, seems to be related to the inactivation of respiratory enzymes.

4) Water Absorption. It has long been found that the potato discs immersed in water absorb to an extent that their volume enlargement becomes considerably appreciable and that for this water absorption, the energy supplied via ATP is available.

Water absorption of sweet potato which morphogenetically belongs to the root is much less than potato which corresponds to the stem.

The sweet potato damaged by the low temperature treatments, not only loses its

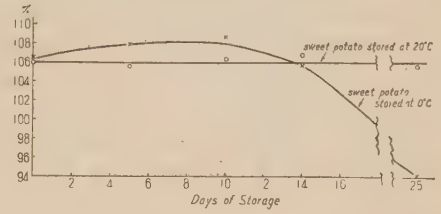


FIG. 5. Water Intake Related to the Duration of Storage.

$$\text{Expressed as: } \% = \frac{\text{Wt. after } H_2O \text{ absorption}}{\text{Wt. before } H_2O \text{ absorption}} \times 100$$

capability of water absorption but also decreases in its weight. This may be attributed to the outflow of components within the cytoplasm. The curves represented in Fig. 5 show that there is a rather decrease in weight at the 25th day. From these curves, however, much cannot be said since experiments have not been extended to the detailed study of the changes which may appear after a 10 day-treatment. The result, in all probability, can provide us with a rough trend of the event.

5) Inoculation of *Rhizopws nigricans*. The sweet potato kept at 0°C for 15 days was readily affected by soft rot caused by

TABLE I

PENETRATION OF *RHIZOPUS NIGRICANCE*

	Sweet Potato						Potato	
Days of Storage	5		9		15		9	
Temperature °C	25	0	25	0	25	0	25	0
(Varieties)								
R102	−	−	−	−	−	##	−	+
Ehrenberg	−	−	−	−	−	##	−	+
Iwasaki	−	−	−	−	−	##	−	−
Unidentified*	−	−	−	+	−	##	−	+

* A vareity isolated by us from a Contaminated sweet potato.

16) J. Bonner et al; *Plant Physiol.*, **24**, 720 (1949).

17) T. Akazawa and I. Uritani; *J. Biochem.* **41**, 631 (1954).

Rhizopus nigricans, whereas the same material kept at 20°C was found to be resistant to the fungus (Table I).

DISCUSSION

The sweet potato stored at 0°C seems to have a critical point at the 10th day or thereabout. At this point, some appreciable changes as follows are observed: a temporary increase in respiration (measured at 25°C) followed by a rapid fall, reduction in the effect of DNP on respiration, an increase in Po accompanied by a concomitant decrease in Pi, and a noticiable rise in pH. These changes seem to be followed by diminution in resistance to the attack of *Rhizopus nigricans*.

There are some attracting studies[18] dealing with the problem of cold resistance in connection with the lipid components in the interior of the cells. The fact that tropical plants containing fats which melt at higher temperatures than those of plants of northern origin, are not resistant to the cold, can be accounted for the readiness of coagulation of the lipid-components.

Although not much as yet has been known about the lipid-components of sweet potato, it has been reported that the content of the components of sweet potato far exceeds that of potato and moreover, that some phosphatides and yalapin are present in the components[19]. Recently, the importance of lipoproteines in cellular particulates such as mitochondria and microsomes is recognized. Therefore it seems possible to set up such a hypothesis that upon exposure to the cold for a long period, the lipid-components in the cells start to coagulate at a certain point in due course of time and the tissues as a whole come to show some specific features of cold damage.

The coagulation of the components leads to the destruction of the cellular structures which, in the long run, results in the coupling of respiratory oxidation to the oxidative phosphorylation carried out at mitochondria will be blocked and cytochrome oxidase will be inactivated in the course of the events. Some results of recent cytochemical studies show that ATP is necessary for ordinary maintenance of morphology and functions of mitochondria.

The retarded formation of ATP caused by low temperature-storage may indirectly have influence on the rate of the breakdown of mitochondria. The fact that the inhibition of the oxidative phosphorylation observed in the sweet potato treated at low temperatures is accompanied by a decrease in Pi cannot be reasonably elucidated at present. In view of the accumulation of organic phosphates conceivably related to the glycolysis, the possibility that fermentation may by under way, will not obviously be ruled out. So, we wish to examine this possibility in the future.

One of the main functions of the plasma membrane is to selectively pass low-molecular materials into and out of the cell. The efficiency of this action is apparently dependent upon the framework of the lipid-containing plasma membrane and availability of ATP. The cell membrane of the sweet potato treated at 0°C for a certain length of time, loses its selective permeability, owing to coagulation of the lipid components in the membrane and the lowered availability of ATP. Such damage of the cell membranes can be qualitatively suspected from the degree of penetration of *Rhizopus nigricans* into the sliced tissues (Table I). The injured tissues seem to provide this fungi with an available source of nutrients.

We sincerely wish to express our appreciations to Prof. Y. Iwasaki of Kyoritsu Women's College for his kind advice and a gift of strains of *Rhizopus nigricans*, and to Mr. T. Akazawa of our laboratory for his participation in the discussion.

18) L.V. Heilbrumn; *Scientific American*, No. 4 (1954).
19) I. Ose; *J. Agr. Chem. Soc. (Japan)*, **9**, 165 (1933).

Short Communication

Paper-Chromatographic Detection of Ribose in the Hydrolyzate of a Hemicellulose of Broad-Bean Seeds

Sir:

Dehulled broad-bean seeds removed from soluble carbohydrates and proteins gave crude holocellulose after treatment with Takadiastase[1].

Now we have found that a hemicellulose fraction can be precipitated as the copper complex, and that the hydrolyzate of this hemicellulose showed the existence of glucose, galactose, xylose, and ribose, as revealed by paper chromatography.

The detection of ribose in the hydrolyzate of a hemicellulose is of great interest. It was not at all expected, for no description has yet been made concerning the occurrence of ribose in hemicellulose. Though the proof was not made directly, we believe our presumption sufficiently justifiable, since the R_F values coincided by three sets of different solvent systems.

Crude holocellulose was obtained from broad beans by extracting with 0.2% sodium hydroxide (to remove sugars and proteins) followed by boiling with water (to dextrinize starch) and treatment with Taka-diastase (to hydrolyze starch). This crude holocellulose was extracted with 5% sodium hydroxide under nitrogen. Hemicellulose A was precipitated by acidifying the alkaline extract to pH 4.4. Addition of 2 volumes of ethyl alcohol to 1 volume of the filtrate from hemicellulose A gave hemicellulose B.

Hemicellulose B was redissolved in 5% sodium hydroxide. Addition of 1 volume each of the Fehling solution A (copper sulfate) and B (Rochelle salt and sodium hydroxide) to 4 volumes of the alkaline solution of hemicellulose B gave voluminous precipitate (hemicellulose B_1-copper complex). This complex was freed from copper and purified by repeating the dissolution in sodium hydroxide and precipitation with the Fehling solution as in the case of hot-water-soluble hemicellulose of soybeans[2].

The purified hemicellulose B_1 showed m.p. 230–250° (decomposition). It could be hydrolyzed quantitatively by boiling with N sulfuric acid for 4 hours or nearly completely by boiling with 2 N sulfuric acid for 2 hours. The purity of this hemicellulose B_1 was thus ascertained by the quantitative yield of reducing-sugar value after hydrolysis.

The hydrolyzate of hemicellulose B_1, obtained by boiling with N sulfuric acid for 4 hours, neutralizing with barium carbonate, and concentrating to a small volume, gave the following R_F values by paper chromatography.

Solvents	BuOH-AcOH		BuOH-C_5H_5N		PhOH	
	Standard	Sample	Standard	Sample	Standard	Sample
Rhamnose	0.57	—	0.65	—	0.59	—
Ribose	0.54	0.55	0.56	0.56	0.59	0.59
Xylose	0.47	0.48	0.52	0.53	0.43	0.43
Arabinose	0.42	—	0.45	—	0.46	—
Glucose	0.36	0.36	0.39	0.40	0.35	0.37
Galactose	0.36		0.35	0.35	0.41	

The solvent systems were as follows. BuOH-AcOH: *n*-butanol-acetic acid-water (4:1:2)

1) S. Kawamura and H. Nakamura, *J. Agr. Chem. Soc. Japan*, **28**, 854 (1954).

2) S. Kawamura *et al.*, This Bulletin, **19**, 69 (1955).

(double development); BuOH-C_5H_5N: *n*-butanol-pyridine-water (3:1:1.5) (double development); and PhOH: phenol-water (4:1) (single development). The reagents used for spraying were aniline hydrogen phthalate and ammoniacal silver nitrate.

These four sugars were extracted separately from the corresponding spots and were determined by the Somogyi method. First, 50 μl of a hydrolyzate (containing about 7 mg sugars) was chromatographed on paper with BuOH-C_5H_5N (2 runs), and the two portions (hexoses and pentoses) were separately extracted with 10 ml water at 100°. The determination of reducing sugars by the Somogyi method gave the molar ratio of hexoses (glucose plus galactose) : pentoses (xylose plus ribose) to be 60:40. Then each extract was again chromatographed, the hexose portion with BuOH-C_5H_5N (3 runs) and the pentose portion with PhOH (1 run); the molar ratio of glucose : galactose was 3.2:1, while that of xylose : ribose was 7:1. Thus the approximate molar ratio of glucose: galactose : xylose : ribose was 46:14:35:5.

Thanks are due to Dr. Ziro Nikuni, professor at Osaka University, for his generous donation of a sample of ribose.

Sin'itirô KAWAMURA
Teiiti NARASAKI

Department of Agricultural Chemistry,
Faculty of Agriculture, Kagawa University

Received September 26, 1956

AUTHOR INDEX TO VOLUME 20, 1956

SUBJECT INDEX TO VOLUME 20, 1956

Journal of the Agricultural Chemical Society of Japan.

(Printed in Japanese)
Vol. 30, No. 10 (1956)

Journal of the Agricultural Chemical Society of Japan.

(Printed in Japanese)
Vol. 30, No. 11 (1956)

PRINTED BY
KASAI PUBLISHING & PRINTING CO.
(Kasai Shuppan Insatsusha)
MINATO-KU, TOKYO